



Neuropathology of Obstructive Sleep Apnoea

A thesis submitted in fulfilment of the requirements for the degree of Doctor of
Philosophy (Laboratory and Clinical Sciences)

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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List of abbreviations

A β	Beta amyloid
AD	Alzheimer's Disease
AHI	Apnoea hypopnoea index
Apo E	Apolipoprotein E
APP	Amyloid precursor protein
BBB	Blood-brain barrier
BMI	Body mass index
BSA	Bovine albumin serum
CA	Cornu Ammonis
CD11b	Cluster of differentiation molecule 11b
CIH	Chronic intermittent hypoxia
CPAP	Continuous positive airway pressure
CSF	Cerebrospinal fluid
DAB	Diaminobenzidine-nickel sulphate
DTI	Diffusion tensor imaging
EC	Entorhinal cortex
FAD	Familial Alzheimer's disease
fMRI	Functional magnetic resonance imaging
GABA	Gamma amino butyric acid
GFAP	Glial fibrillary acidic protein
GS	Glutamine synthetase
h	Hour
H ₂ O ₂	Hydrogen peroxide
IH	Intermittent hypoxia
IL	Interleukin
LC	Locus ceruleus
MBP	Myelin basic protein
MCI	Mild cognitive impairment
MMSE	Mini mental state examination
mPFC	Medial prefrontal cortex

MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NaOAc	Sodium acetate
NFT	Neurofibrillary tangle
NMDA	N-methyl D-aspartic acid
NREM	Non-rapid eye movement
NSE	Neuron specific enolase
ODI	Oxygen desaturation index
OSA	Obstructive sleep apnoea
OTC	Occipitotemporal cortex
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PSG	Polysomnography
RBD	REM sleep behaviour disorder
REM	Rapid eye movement
SDB	Sleep disordered breathing
SWS	Slow wave sleep
TNF α	Tumour necrosis factor alpha
WM	White matter

Abstract

Obstructive sleep apnoea (OSA) is a sleep disorder involving frequent cessations of breathing due to collapse of the upper airway, leading to sleep fragmentation and hypoxia in the brain. Neuroimaging studies have found evidence of atrophy and degenerative changes in the brains of untreated OSA patients as well as cognitive deficits. Continuous positive airway pressure (CPAP) is an effective treatment that restores airflow when used during sleep. The precise neuropathological changes occurring in the brains of OSA patients are currently unknown. Animal models of intermittent hypoxia (IH) have been utilised to investigate neuropathological changes that might be occurring in the human brain. Such studies have found that mice exposed to IH show brain atrophy, reduced myelination, reactive gliosis and increased phosphorylation of tau protein leading to neurofibrillary tangle (NFT) formation. Additionally, genetically modified mice that express phenotypic changes associated with Alzheimer's disease (AD) and exposed to IH show an increase in a hallmark pathological marker of AD: beta amyloid ($A\beta$) plaques. $A\beta$ plaques and NFTs are abnormal protein aggregates that accumulate in the brains of patients with AD, initially in the hippocampus. One study has previously investigated the neuropathological changes in OSA patients, however the hippocampus was not examined. The aim of the present thesis was to investigate neuropathological changes in *post-mortem* hippocampal tissue of OSA patients using immunohistochemistry. The study also compared the similarity of these changes to those observed in AD, as well as the effect of CPAP treatment and the effect of age. The study sample included brain tissue from 34 (18 female, 16 male) Icelandic OSA patients with a mean age of 67.0 ± 11.1 years.

AD is typically diagnosed in late life, with age being the biggest risk factor, but neuropathological changes are thought to begin decades prior to clinical diagnosis. OSA is usually diagnosed in midlife. Since there is a higher prevalence of OSA among patients with dementia/AD than in the general elderly population, it seems possible that OSA may trigger the neuropathological changes associated with AD. In this thesis, the first project assessed the accumulation of $A\beta$ plaques and NFTs in the brainstem and hippocampus of OSA patients. In the brainstem, no association was found between the presence of $A\beta$ plaques or NFTs and severity of OSA. In the hippocampus, significant relationships were found between OSA severity and the burdens of NFTs ($r^2=0.166$, $p=0.017$) and $A\beta$ plaques ($r^2=0.134$, $p=0.033$). The burdens of NFT and $A\beta$ in the hippocampus increased with OSA severity, with both relationships being stronger in patients who did not use CPAP and weaker in those using

CPAP (NFTs No CPAP vs CPAP, $r^2=0.315$ vs 0.023 ; A β No CPAP vs CPAP, $r^2=0.360$ vs 0.059). This result indicates that CPAP is protective against the accumulation of A β plaques and NFTs. Age was a factor in the relationship between OSA severity and NFTs ($r^2=0.314$, $p=0.001$), but not for A β burden.

The second project investigated the relationship of OSA severity and reactive gliosis. Reactive gliosis is the response of glial cells to injury in the brain and is commonly seen after a variety of different brain injuries including AD. Reactive gliosis was assessed via counts of immunolabelled microglia and astrocytes and by measuring the intensity of immunoreactivity in five regions of the hippocampus: hilus, CA3, CA1, subiculum and entorhinal cortex. Evidence of reactive gliosis in relation to OSA severity was found in all regions except the CA1, with age influencing some relationships. Increasing OSA severity was associated with increased numbers of microglia in the hilus ($r^2=0.134$, $p=0.033$) and subiculum ($r^2=0.197$, $p=0.010$), and increased numbers of astrocytes in the subiculum ($r^2=0.132$, $p=0.038$), increased intensity of immunolabelling of astrocytes in the entorhinal cortex ($r^2=0.178$, $p=0.028$) and decreased intensity of immunolabelling of astrocytes in the CA3 ($r^2=0.123$, $p=0.042$). These findings indicate that reactive gliosis is present in the hippocampus in severe OSA and it involves regional subpopulations of astrocytes. All significant relationships between glial markers and OSA severity were stronger among CPAP non-users and most were weaker among CPAP users, suggesting that CPAP may attenuate these changes.

The third project investigated the size of the hippocampus and degree of demyelination in relation to OSA severity. Severe hippocampal atrophy and demyelination are commonly seen in AD and neuroimaging studies suggest that similar changes may occur in OSA. Cortical cell layer thickness was measured in the CA1 and entorhinal cortex, and layer area was measured in the dentate gyrus. The intensity of myelin basic protein immunoreactivity was measured in the same regions to investigate demyelination. As OSA severity increased, decreases were seen in the thickness of cortical layers in CA1 (total depth, $r^2=0.135$, $p=0.039$; layer 1, $r^2=0.157$, $p=0.025$; layer 2, $r^2=0.255$, $p=0.003$; layer 3, $r^2=0.185$, $p=0.014$), the molecular layer of the dentate ($r^2=0.136$, $p=0.038$) and to a lesser extent in entorhinal cortex (layer 1, $r^2=0.186$, $p=0.028$). Cortical thickness in layer 2 of CA1 was influenced by age. As OSA severity increased, the extent of myelin diminished in the entorhinal cortex (layer 6, $r^2=0.282$, $p=0.006$; deep white matter, $r^2=0.390$, $p=0.001$), indicating that demyelination occurred in these regions. The extent of myelination was unaffected by age. Many of the hippocampal size relationships were stronger in CPAP non-users and weaker in CPAP users,

suggesting that protection is afforded to CPAP users. Conversely, most of the demyelination relationships did not change with CPAP status, indicating that CPAP is ineffective at attenuating demyelination.

For the first time, AD-like neuropathology, reactive gliosis, cortical atrophy and demyelination have been observed in the hippocampus of OSA patients. Additionally, CPAP has been shown to reduce such neuropathological changes, providing strong incentives for patients to adhere to treatment. The spatiotemporal pattern of neuropathological changes in OSA is remarkably similar to that seen in early AD, suggesting that OSA may accelerate the pathogenesis of AD. The present thesis has limitations, including the relatively small sample size and the lack of a control group, and additional research will be needed to confirm and extend these findings. Nonetheless, this thesis has found that there are significant neuropathological changes associated with increasing OSA severity that can be attenuated with CPAP treatment, and it supports the interesting possibility that CPAP may have potential as a prophylactic treatment against AD.

Chapter 1 Literature review

1.1 Sleep

Humans spend about one third of their lives asleep. There are two types of sleep: non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep; NREM sleep has four stages (stages 1- 4). Stages 1 and 2 are the initial sleep stages and are considered light sleep. People can be easily woken from these stages and sometimes are unaware they were asleep. Stages 3 and 4 are considered deep or slow wave sleep (SWS). It is much harder to wake a person from stage 3 or 4 sleep (Rama, Cho, & Kushida, 2005). REM sleep is so named because of the notable eye movements occurring during this stage. The amount of time spent in each stage changes during the course of the night (Figure 1.1). More time is spent in deep sleep (stages 3 and 4) earlier in the night whereas closer to waking there is less stage 3 and 4 sleep and more REM sleep.

<Image removed due to copyright restrictions>

Figure 1.1 Typical sleep patterns of young adults (top) and elderly adults (bottom). Source: “Sleep problems in the elderly” by D. N. Neubauer, 1999, *American Family Physician*, 59 (9), 2551-2558.

1.1.1 Sleep across the lifespan

There is still much debate about the precise amount of sleep needed per night; however there is consensus regarding variations in the quantity of sleep across the lifespan. Generally, total sleep time decreases with increasing age. Infants sleep for approximately 16 hours in a 24 hour period, children sleep for around 12 hours and adolescents and adults function well with about 8 hours, however there is large individual variability and the amount of sleep required for optimal function during wakefulness varies between individuals (Rama et al., 2005).

It is unclear whether elderly people sleep less than younger adults. While a meta-analysis of sleep across the lifespan found that elderly people sleep less than younger adults (Ohayon, Carskadon, Guilleminault, & Vitiello, 2004), this study did not account for daytime napping which has been shown to increase in the elderly (Ayalon & Ancoli-Israel, 2005). Therefore it may be that while nighttime sleep decreases, the total sleep time across a 24 h period may not differ significantly, due to a redistribution of sleep across the 24 h period with increasing age (Ayalon & Ancoli-Israel, 2005). As well as changes in sleep time distribution, there are also architectural changes to sleep as we age. Babies and children experience more REM sleep and spend more time in sleep stages 3 and 4 than adults (Sheldon, 2005). Older people experience less deep, restorative sleep (stages 3 and 4) and more light sleep (stage 1), more nighttime awakenings and increased daytime sleepiness than younger adults (Figure 1.1) (Ayalon & Ancoli-Israel, 2005; Neubauer, 1999). A meta-analysis of sleep across the lifespan found increases in the percentage of stage 1 and 2 sleep and the number of nighttime awakenings as well as decreases in the percentage of slow wave sleep and REM sleep, sleep efficiency and total sleep time (Ohayon et al., 2004).

There are physiological reasons that may contribute to sleep changes in the elderly. Changes in serum melatonin levels are a physiological reason for sleep changes. Melatonin is a sleep promoting hormone released into the bloodstream at night, with levels peaking around 3AM (Claustrat, 2003). Melatonin levels have been shown to reduce to almost the same as wakefulness levels in people aged over 70 years (Karasek, 2004). Other changes that may disrupt the circadian rhythm include reduced exposure to bright light and physical activity and neuronal degeneration of the suprachiasmatic nucleus, the site of the circadian pacemaker (Edwards et al., 2010). Whether these changes are the result of normal ageing or an underlying pathology is not clear. Many of the sleep disruptions seen in old age are associated with comorbidities and subsequent pharmacological treatments for those

comorbidities can also affect sleep quality (Foley, Ancoli-Israel, Britz, & Walsh, 2004).

There are many reasons for changes in sleep patterns as we age, and many consequences of those changes.

1.1.2 Sleep disruption and memory impairments

Sleep is necessary for many cognitive functions and sleep deprivation has been associated with negative mood states, and impaired cognitive and motor performance (Goel, Rao, Durmer, & Dinges, 2009). Cognitive deficits can be detected in people with sleep deprivation (total sleep loss 1-2 nights), sleep restriction (sleep time reduced to 4 or 6 hours per night) and sleep fragmentation (disrupted sleep continuity without reduced sleep time), in young adults as well as in older people who have naturally fragmented or disrupted sleep (Scullin & Bliwise, 2015).

Changes to sleep patterns affect learning and memory. It is generally accepted that sleep aids in the consolidation of memory, as evidenced by studies showing reduced memory retrieval after sleep deprivation and improved memory retrieval after a night's sleep or even just a nap (Scullin & Bliwise, 2015). Therefore, people who experience sleep deprivation, restriction or fragmentation, such as those with sleep disorders, are at risk of impaired memory, which indeed is seen in many sleep disorders. More concerning is the knowledge that people who experience sleep restriction or fragmentation may be less aware of their reduced capabilities than people who are sleep deprived. Van Dongen and colleagues (2003) found the same degree of impairment on the psychomotor vigilance task in subjects who had been sleep deprived for three days and those who had been sleep restricted (4 h per night) for 14 days. They also found a large discrepancy between subjective sleepiness scores in the two groups, with sleep deprived individuals reporting high sleepiness scores and sleep restricted participants reporting significantly lower sleepiness scores. This study shows that subjective sleepiness is not a reliable measure of cognitive deficit after sleep restriction. While this study did not investigate memory, animal studies have shown deficits in spatial memory and memory consolidation after sleep disruption (McCoy et al., 2013; Rolls et al., 2011). Further, impairments in fear conditioning memory and short-term memory have been found in sleep deprived human subjects (Chengyang et al., 2016; Menz et al., 2013). These studies highlight the importance of sleep in memory processing. As well as memory deficits, lack of sleep can affect brain regions associated with memory, particularly the hippocampus.

1.1.3 Sleep disruption and neuropathological changes in the hippocampus

As well as cognitive consequences, sleep modifications have been shown to have structural neurological consequences. Many brain regions have been shown to have reduced activity after sleep deprivation (Dang-Vu, 2014). The hippocampus is frequently shown to be vulnerable to sleep disruption and this correlates with the memory impairments seen (Joo, Kim, Suh, & Hong, 2014; McDermott et al., 2003). Reduced connectivity between the hippocampus and the precuneus, post-central gyrus and parts of the frontal, temporal and occipital lobes as well as short term memory impairments were found after sleep deprivation (Chengyang et al., 2016). It has been shown that the volume of the hippocampus is reduced after sleep deprivation and this may be due to hippocampal injury combined with reductions in neurogenesis (the production of new brain cells) (Kreutzmann, Havekes, Abel, & Meerlo, 2015). For instance, mice that were sleep deprived for 2 days had fewer viable cells and more degenerative morphological changes in their hippocampus compared to controls (Chittora, Jain, Prasad, & Bhatnagar, 2016). A sleep fragmentation protocol in rats reported a 32% reduction in the number of proliferating cells in the dentate gyrus of the hippocampus (Sportiche et al., 2010). This study also showed that the reduction in cell proliferation was correlated with impairment on a cognitive task. This finding underlines the importance of sleep in protecting vulnerable brain regions from degeneration. As sleep changes and cognitive deficits are more common in older people than younger people, it is necessary to understand the relationship between sleep and neurodegeneration, an increasingly common condition of old age.

1.1.4 Sleep in neurodegenerative conditions particularly Alzheimer's disease

There are changes in sleep associated with healthy ageing as mentioned above, however sleep disruption in neurodegenerative disorders is often more severe than that seen in healthy peers. Many changes in sleep patterns are associated with Alzheimer's disease (AD) including early rising, increased daytime napping and excessive daytime sleepiness (Vitiello, 2005). While these changes are similar to those seen in the general elderly population, they are more prevalent and pronounced in AD. People with AD have reduced stage 3 and 4 sleep, less total sleep time, less REM sleep and more awakenings compared to age-matched controls (Vitiello & Borson, 2001; Vitiello, Poceta, & Prinz, 1991). The extent of the changes in sleep architecture is correlated with the severity of AD (Vitiello et al., 1991). A study of AD transgenic mice that had been subjected to chronic mild sleep restriction found increased memory impairments compared to transgenic AD mice that had not been subjected to sleep restriction (Rothman, Herdener, Frankola, Mughal, & Mattson, 2013). Further, this study

showed an increase in the accumulation of the key AD proteins, beta amyloid and phosphorylated tau, in the sleep restricted group. This outcome suggests a relationship between sleep quality and AD, although which comes first remains a matter of debate.

There are also increases in the prevalence of specific sleep disorders in neurodegenerative disorders. Neurodegenerative diseases that are associated with increased alpha synuclein (often termed Lewy body diseases), such as Parkinson's disease and multiple system atrophy, have clear associations with REM sleep behaviour disorder (RBD) (Suescun, Ellmore, & Schiess, 2016). RBD is characterised by acting out dreams due to lack of inhibitory controls during REM sleep. The prevalence of RBD is estimated at 0.5% of the population (Howell & Schenck, 2015), with 81% of those affected converting to a Lewy body disease in their lifetime (Schenck, Boeve, & Mahowald, 2013). In particular, the risk of developing Parkinson's disease is 8.38-fold greater for those with RBD (Y. Xu, Yang, & Shang, 2016). RBD is usually diagnosed years or decades prior to the development of a Lewy body disease and has been suggested to be a prodromal stage of this type of neurodegeneration (Suescun et al., 2016). The fact that sleep disruptions are seen many years prior to disease diagnosis suggests that sleep disorders could have potential as early diagnostic markers of neurodegenerative disease. AD has also been associated with an increased prevalence of a sleep disorder, obstructive sleep apnoea (OSA). It has been found that 49-63% of people with AD/dementia also have OSA or sleep disordered breathing (SDB) (Gehrman et al., 2003; K. M. Rose et al., 2011). This is more than twice the prevalence of OSA in the healthy elderly population, which has been estimated at approximately 20% (Young, Shahar, et al., 2002). It remains to be seen whether sleep disorders cause neurodegeneration or whether neurodegeneration causes sleep disorders; alternatively both conditions could affect the other, in a positive feedback system, as proposed by Mattis & Sehgal (2016). Regardless of the direction of the causal relationship, it is important to identify sleep disorders early, with the aim to improve or slow the progression of neurodegeneration.

1.2 Alzheimer's disease

Alzheimer's disease is a neurodegenerative dementia that commonly occurs in old age. There are two types of AD: the less common and genetically inherited, familial AD (FAD) and the

more common, sporadic AD. For the purpose of this review “AD” will refer to sporadic AD unless otherwise stated. The first clinical signs of AD are forgetfulness and confusion, which progress into more serious cognitive problems (Burns & Iliffe, 2009). Specifically, a continual degradation of episodic memory and an inability to form new memories is the major clinical symptom of AD. Other symptoms include executive dysfunction and attention deficits; behavioural and psychological symptoms are also common (Burns & Iliffe, 2009; Cerejeira, Lagarto, & Mukaetova-Ladinska, 2012). Many people who have AD progress slowly through a state of mild cognitive impairment (MCI) until they are diagnosed with probable AD. This diagnosis can only be confirmed upon autopsy of the brain to reveal the presence of beta amyloid ($A\beta$) plaques and neurofibrillary tangles (NFTs); the distinguishing neuropathological features of AD (discussed further below) (Braak & Braak, 1991). There are many other neuropathological changes associated with AD, some of which will be discussed below.

There is not a strong genetic predisposition to sporadic AD, however the genetic variants of the gene encoding for apolipoprotein E (Apo E) have been shown to alter the likelihood of disease development and progression (J. Kim, Basak, & Holtzman, 2009). Apo E has also been implicated in increasing the risk of conversion from MCI to AD (Fei & Jianhua, 2013). MCI does not always progress to AD or dementia and the factors affecting this progression are wide and varied (R. Roberts & Knopman, 2013). The rate of conversion from MCI to dementia/AD has been estimated at 10-15% per year (R. Roberts & Knopman, 2013). Risk factors for conversion of MCI to dementia/AD include diabetes, metabolic syndrome, low dietary folate, neuropsychiatric symptoms and multi-domain MCI compared to single cognitive domain MCI (Aretouli, Okonkwo, Samek, & Brandt, 2011; Cooper, Sommerlad, Lyketsos, & Livingston, 2015; R. Roberts & Knopman, 2013).

There is no known cure for AD and current treatments are inadequate. While some pharmacological approaches are approved for treatment and slow cognitive deterioration for a period, many patients experience quicker cognitive decline after the treatment ends (Burns & Iliffe, 2009). Treatment with acetylcholinesterase inhibitors or memantine (an NMDA receptor antagonist) may be useful in treating cognitive symptoms in some patients for a short time, but they do not address the underlying pathology of the disease (Jackson, 2014). Studies attempting to remove one of the underlying pathological markers from the brain, using anti-amyloid antibodies have not provided conclusive results, with most studies reporting no cognitive improvements compared to placebo treatment (Brody et al., 2016; Doody et al.,

2014; Karran & Hardy, 2014; Vandenberghe et al., 2016). Some studies have shown small cognitive improvements for the treatment of mild AD, with one particular anti-amyloid drug, solanezumab (Rygiel, 2016; Siemers et al., 2016). One recent study has shown reduced A β in the brain via positron emission topography (PET) imaging with a different amyloid antibody, aducanumab (Sevigny et al., 2016). However, this was a small trial only conducted in prodromal or mild AD patients. The ineffectiveness of most treatments suggests that AD is a complex disease and the causative factors are likely to be in play decades before clinical symptoms are exhibited. Much research is being conducted to uncover the as-yet-unknown causative agent in AD in the hope that this will lead to more effective treatments.

1.2.1 Neuropathological changes in AD

The numerous neuropathological changes associated with AD will be discussed in detail in this section. Overall, significant brain shrinkage is seen in AD with as much as 18% reduced brain volume compared to age matched healthy controls (Miller, Alston, & Corsellis, 1980) and a 40% increase in ventricular volume (De la Monte, 1989). There seems to be no difference in the rate of grey and white matter loss, however there are regional differences in the atrophy seen (Mann, 1991). Grey matter loss is seen in the medial temporal lobe, cingulate gyrus, insular cortex, caudate nucleus and frontal cortex of AD patients (Baron et al., 2001; Frisoni et al., 2002; Karas et al., 2004). A study investigating the dynamic loss of grey matter in AD found that a significant loss in the temporal and entorhinal cortices before significant loss was detected in the frontal, parietal and occipital cortices (Thompson et al., 2003). Further, this study demonstrated significant lateralisation, with greater loss seen in left cingulate and temporal cortices than right. Interestingly, but perhaps not surprisingly, a sparing of grey matter loss was seen in the primary sensory and motor regions. The dynamic grey matter loss in this study was correlated with cognitive decline on the mini mental state examination (MMSE), a common cognitive assessment of dementia, and corresponded to the pattern of neuropathological accumulation of NFTs in AD (Braak & Braak, 1991).

Degeneration of major white matter pathways in the brain has also been seen in AD.

Kitamura and colleagues (2013) found pathological white matter alterations in three white matter tracts, uncinate fasciculus (connects temporal lobe structures with orbitofrontal cortex), inferior longitudinal fasciculus (connects temporal and occipital lobes) and the inferior occipito-frontal fasciculus (connects occipital and frontal lobes). These three tracts provide connections between areas that also see significant grey matter loss and neuropathological damage in AD. Interestingly, recent research suggests that damage to the

white matter tracts precedes the grey matter volume loss (Kehoe, McNulty, Mullins, & Bokde, 2014). White matter degradation in AD will be discussed in more detail below.

The hippocampus, located in the medial part of the temporal lobe, is the focal point of neuropathology in AD; its main function is memory processing and consolidation, the characteristic cognitive deficit of AD. Further, the hippocampus and surrounding cortical tissue is where cellular neuropathology, specifically A β plaques and NFTs, first appear. Cerebral atrophy in the temporal lobe is seen earlier and is more severe than atrophy in other areas. Moreover, the volume of the medial part of the temporal lobe is reduced in patients with MCI (Karas et al., 2004). Severe reductions in volume are seen in the medial temporal lobe, including hippocampus, of AD patients as well as the parietal and cingulate cortices (Karas et al., 2004; Rombouts, Barkhof, Witter, & Scheltens, 2000). The volume of the hippocampus has been found to be reduced by 28% in AD patients compared to healthy age-matched controls (Mann, 1991). This reduction is likely due to a loss of neurons and their axons, meaning that both grey and white matter are affected. The neuropathological features that are associated with AD are discussed below.

1.2.1.1 Beta amyloid

The most studied area of AD neuropathology is the accumulation of the protein beta amyloid (A β). A β is a naturally occurring protein in the brain, however little is known about its normal function. A β is produced by the cleavage of the amyloid precursor protein (APP). The production of A β from APP depends on the site at which APP is cleaved. If α -secretase cleaves APP it does so in the middle of the A β peptide site and therefore no A β is produced (Swerdlow, 2007). If β - and γ -secretase cleave APP then a 40 or 42 amino acid length A β peptide is produced (A β 40 or A β 42 respectively) (Swerdlow, 2007). The A β peptides are single proteins or monomers; however they tend to group together to form oligomers. Soluble A β oligomers tend to aggregate with each other and become amyloid fibrils, these fibrils tend to aggregate to form the A β plaques that are a characteristic feature of AD (Glabe, 2008; Kaye & Lasagna-Reeves, 2012). The factors affecting which enzyme cleaves APP and whether or not A β is produced are yet to be fully understood, except for in FAD. FAD occurs due to a gene mutation in the gene that codes for APP or presenilin. These gene mutations lead to the cleavage of APP predominantly at the β and γ secretase sites, leading to substantial A β plaque deposition by as early as 50 years of age (Robakis, 2015; Shepherd, McCann, & Halliday, 2009). A β production in sporadic AD does not occur via a gene

mutation; however the pattern of accumulation of A β is the same in both sporadic and familial AD.

The pattern of accumulation of A β plaques in AD has been thoroughly investigated (Braak & Braak, 1991, 1997; Thal et al., 2000). It is well established that plaque accumulation begins in the inferomedial part of the temporal lobe, specifically at the collateral sulcus. From the collateral sulcus, plaques can be seen to spread into the entorhinal cortex, subiculum and then hippocampus proper (Thal et al., 2000). In the later stages of the disease, plaques can also be seen in other parts of the temporal lobe as well as the frontal and occipital lobes. A β plaques increase in density in these regions and accumulate in most other cortical regions, as well as in basal ganglia structures (Braak & Braak, 1991). Regions adjacent to the central sulcus, including the primary motor and sensory cortices, tend to have lower plaque burdens (Braak & Braak, 1991).

While the A β plaques were the first type of A β identified as a feature of AD, the soluble A β oligomers that aggregate into fibrils and then plaques have been investigated in recent times. The deposition of A β plaques correlates poorly with cognitive deficits in AD (Samuel, Masliah, Hill, Butters, & Terry, 1994; Terry et al., 1991), the quantity of soluble A β oligomers may correlate well with cognitive deficits; however there is no direct evidence of this. Earlier studies in humans found correlations between soluble A β levels and disease severity, as measured by decreasing synaptic density (Lue et al., 1999) and the accumulation of NFTs (another neuropathological feature of AD, discussed below) (McLean et al., 1999). Both decreasing synaptic density and the accumulation of NFTs correlate well with cognitive deficits in AD (Samuel et al., 1994; Terry et al., 1991). However, this correlation alone cannot substantiate the claim that soluble A β levels correlate with cognitive decline. A more recent study found a correlation between cognitive decline in AD and so called ‘soluble fibrillar oligomers’ of A β with no correlation between cognition and ‘prefibrillar oligomers’ of A β (Tomic, Pensalfini, Head, & Glabe, 2009). However, the exact A β aggregate being detected in this study is uncertain as the terms ‘soluble fibrillar oligomer’ and ‘prefibrillar oligomer’ were created by the authors with no precise definitions, therefore the relationship with cognitive decline should be interpreted with caution. Despite the lack of direct evidence to suggest a relationship between soluble A β oligomers and cognitive decline in humans with AD, animal models have found that soluble A β correlates with spatial memory deficits, whereas insoluble A β , total A β and A β plaque numbers do not (W. Zhang et al., 2011). This suggests that soluble A β oligomers are associated with toxicity and insoluble plaques may be

a compensatory mechanism of the brain to decrease the amount of toxic oligomers present. Sengupta and colleagues (2016) suggest that there is an inverse relationship between the increasing size of A β aggregates from oligomer to fibrils and decreasing toxicity of the A β species. Such that, oligomers made up of fewer A β proteins are more toxic than larger oligomers, which are in turn more toxic than large, insoluble A β fibrils and plaques.

It is not known whether A β accumulation is due to an increase in the β - and γ -secretase cleavage of APP pathway or a lack of clearance of A β from the brain. A β is thought to be cleared from the brain at a rate of approximately 7-8% per hour, based on measurements of both cerebrospinal fluid (CSF) and venous blood in healthy adults (Bateman et al., 2006; K. F. Roberts et al., 2014). In AD, the rate of A β clearance is reduced to approximately 5% per hour (Mawuenyega et al., 2010). Various mechanisms are involved in clearing A β from the brain including phagocytosis by glial cells, proteolytic degradation and transportation to the venous blood supply either via the CSF or directly across the blood-brain barrier (BBB) (Baranello et al., 2015; Bates et al., 2009; C. Y. D. Lee & Landreth, 2010; K. F. Roberts et al., 2014). Reduced clearance of A β from the brain in AD could be due to a multitude of factors affecting one or more of these mechanisms. Of interest, it has recently been shown in mice that sleep can increase the clearance of A β from the brain via the CSF (L. Xie et al., 2013). Moreover, mild sleep restriction in AD mice was found to increase the accumulation of A β in the brain (Rothman et al., 2013), possibly due to a decreased clearance of A β . This finding raises the possibility that people who receive inadequate sleep, such as in OSA, may be more susceptible to decreased A β clearance, which could potentially lead to increased A β accumulation and AD.

Additionally, it has been found that the relationship between NREM slow wave sleep and hippocampus-dependent memory consolidation in humans is significantly influenced by A β in the medial prefrontal cortex (mPFC) (Mander et al., 2015). This study used PET-A β imaging to determine the A β burden in mPFC of healthy older participants; however the study failed to account for any A β in the hippocampus. Given that the study measured hippocampus-dependent memory consolidation and it is known that A β accumulates in the hippocampus and the surrounding inferior temporal region at the same disease stage as mPFC A β accumulation (Braak & Braak, 1991, 1997), it seems necessary to account for any A β deposition in the hippocampal region. Deposition of A β in the hippocampus may contribute to the relationship between hippocampal-dependent memory consolidation and NREM slow

wave sleep. Alternatively, an additional mechanism may both prevent efficient sleep and increase A β accumulation.

It has long been believed that A β is the causative agent in AD and the amyloid cascade hypothesis of AD has been developed to explain this relationship (Hardy & Higgins, 1992). This hypothesis states that the deposition of A β leads to all of the other changes associated with AD (Hardy & Selkoe, 2002; Selkoe & Hardy, 2016). This hypothesis fits well with the progression and development seen in FAD, however, it has more difficulty accounting for the changes seen in sporadic AD, such as the presence of NFTs prior to A β deposition, the lack of correlation between A β deposition and cognitive deficits, and the lack of a treatment effect with anti-amyloid drugs (Braak, Thal, Ghebremedhin, & Del Tredici, 2011; Doody et al., 2014; Kepp, 2017; Vandenberghe et al., 2016). Further, A β is normally produced in small quantities, so to see an accumulation of A β in the brain there needs to be an increase in the production of A β or a decrease in clearance of A β from the brain. An increase in production occurs when the cleavage pathway changes from α -secretase predominant to β - and γ -predominant (Swerdlow, 2007). Multiple mechanisms of decreased A β clearance have been suggested, as discussed above. Either situation suggests that there is an earlier event/s triggering a change in A β production or degradation. Therefore, as suggested by Robinson and Bishop (2002) (Bishop & Robinson, 2002) A β accumulation appears likely to be a downstream product of another causative agent.

1.2.1.2 Neurofibrillary tangles

Neurofibrillary tangles (NFTs) are a pathological hallmark of AD, although they occur in other neurodegenerative conditions as well. NFTs are made up of hyperphosphorylated tau protein. Tau is a cytoskeletal protein found in neurons in the brain and it is essential for stabilising axonal microtubules. Put simply, it maintains the structural integrity of the cell. If tau becomes hyperphosphorylated, it forms paired helical filaments due to protein misfolding. The accumulation of paired helical filament tau inside neurons leads to the formation of NFTs and eventual neuronal death (Grundke-Iqbal et al., 1986; Köpke et al., 1993; Takashima, 2015). NFTs are resistant to proteolysis and after the death of their neuron, they persist as a ‘ghost’ or ‘tombstone’ tangles. It is not known whether ghost tangles are eventually degraded by macrophages or glial cells (Braak & Braak, 1991).

NFTs accumulate in the brain in AD in a similar spatiotemporal pattern to A β plaques (Braak & Braak, 1991, 1997). They begin at the collateral sulcus and then spread from this area to

adjacent cortical regions (Braak, Alafuzoff, Arzberger, Kretschmar, & Tredici, 2006). The presence of NFTs is better correlated with cognitive decline in AD than A β accumulation (Samuel et al., 1994).

It is not known what causes the hyperphosphorylation of tau that leads to NFT formation. Increases in phosphorylated tau have been reported in response to oxidative stress (Su et al., 2010), neuroinflammation (Padmanabhan, Levy, Dickson, & Potter, 2006), chronic stress (L. F. Zhang et al., 2012), reduced mitochondrial transport (Iijima-Ando et al., 2012), hypoxia (Fang, Zhang, Meng, Du, & Zhou, 2010; C. E. Zhang et al., 2014), ischaemia (Wen, Yang, Liu, & Simpkins, 2004) and traumatic brain injury (Tran, LaFerla, Holtzman, & Brody, 2011). Further, it has been suggested that A β oligomers can stimulate the phosphorylation of tau, at least in neuronal cultures (De Felice et al., 2008). Some research groups however, suggest that phosphorylated tau and NFTs are present before extracellular A β deposits are seen (Braak & Del Tredici, 2015). The primary causative agent of AD seems yet to be determined, since the amyloid cascade hypothesis is unable to account for all of the findings associated with AD initiation and progression.

Evidence suggests that sleep may aid in the prevention of abnormal tau production, with one study finding significantly more phosphorylated tau in the brains of sleep-restricted AD mice compared to controls (Rothman et al., 2013). However, a more recent study found a decrease in phosphorylated tau in sleep-restricted mice compared to controls but found an increase in insoluble tau (Di Meco, Joshi, & Praticò, 2014). Di Meco and colleagues (2014) suggested that sleep disruption leads to conformational changes in the tau protein that may have prevented phosphorylation and accelerated insoluble aggregation. Although these two studies obtained differing results, they both demonstrated that sleep restriction alters the functioning of tau in mice, and this fact provides provisional support for the idea that people with sleep disorders may be more susceptible to neurodegenerative changes, such as NFT accumulation.

1.2.1.3 Neuronal and synaptic loss

Neuronal loss is a significant factor in the neuropathology of AD. As well as generalised brain atrophy, some regions suffer more severe deterioration due to the loss of specific neuronal populations. For instance, cholinergic neurons of the basal forebrain experience

significant loss in AD compared to healthy controls (Ferreira-Vieira, Guimaraes, Silva, & Ribeiro, 2016; Whitehouse et al., 1982). Atrophy of the basal forebrain occurs at an increased rate compared to whole brain atrophy (Grothe, Heinsen, & Teipel, 2013). This finding has been linked to cognitive deficits because the cholinergic neurons in this region, specifically in the nucleus basalis of Meynert, are associated with memory functioning (Hasselmo, Anderson, & Bower, 1992). Further, atrophy of the basal forebrain correlates with decreasing spatial navigation skills (Kerbler, Nedelska, et al., 2015) and the accumulation of A β (Kerbler, Fripp, et al., 2015) in AD patients. Parietal lobe (Grignon, Duyckaerts, Benneicib, & Hauw, 1998) temporal lobe, entorhinal cortex and hippocampus (Gómez-Isla et al., 1997; Gómez-Isla et al., 1996) also experience significant neuronal loss in AD. Regions of the hippocampus, particularly CA1, seem to be especially susceptible to neuronal loss in AD, which is thought to account for much of the volumetric loss in the hippocampus (Kril, Hodges, & Halliday, 2004; West, Coleman, Flood, & Troncoso, 1994). Neuronal loss in AD is attributed to the formation of NFTs; however, the rate of neuronal loss exceeds NFT development indicating that there are other causes of neuronal death (Gómez-Isla et al., 1997; Kril, Patel, Harding, & Halliday, 2002).

A β is thought to be responsible for some of the neuronal death seen in AD. This has been suggested to occur by two mechanisms; an increase in toxic A β leading to apoptosis and/or a decrease in normal A β in cells. A β is toxic to cultured neurons at high concentrations (Yankner, Duffy, & Kirschner, 1990) and it is thought that the increase in extracellular A β in AD leads to downstream neuronal death via apoptotic mechanisms (Selkoe & Hardy, 2016). However, while there is evidence of A β -induced apoptosis in neuronal culture models (Yankner et al., 1990), there is little evidence of apoptosis occurring in human AD brains. Conversely, there is also evidence to suggest that a decrease in the normal functioning of A β contributes to neuronal loss by impairing synaptic functioning. Normally, A β aids in the regulation of vesicle release at the synapse (Abramov et al., 2009) and APP has been shown to be important in synaptic plasticity (X. Zhang et al., 2013). The aggregation of A β into plaques in AD limits the availability of A β to aid in synaptic functioning. Indeed, lack of endogenous A β results in significant memory impairments due to reduced long term potentiation (Morley et al., 2010) and neuronal death (Plant, Boyle, Smith, Peers, & Pearson, 2003).

A significant number of synaptic connections are lost in AD, particularly in the hippocampus (Scheff, Price, Schmitt, & Mufson, 2006) and this correlates significantly with cognitive

decline (Samuel et al., 1994; Terry et al., 1991). Coleman and Yao (2003) suggest that there are still living neurons with reduced synaptic connections, particularly at post-synaptic dendrites present in AD (Buell & Coleman, 1979). This is supported by the relationship between synaptic loss and NFT development. Progressive synaptic loss occurs with increasing accumulation of intraneuronal phosphorylated tau, leading to eventual neuronal death (Merino-Serrais et al., 2013). It is likely that synaptic loss occurs downstream of the primary neuropathological changes, including A β and NFT deposition, and prior to neuronal death (Lu, Nagappan, Guan, Nathan, & Wren, 2013). This is supported by the results of an animal study in which significant synaptic and neuronal loss is only seen in transgenic animals exhibiting both A β and NFT phenotypes (Saul, Sprenger, Bayer, & Wirths, 2013). Either pathology on its own is not sufficient to produce the same degree of synaptic loss as both together. Further, A β oligomers have been associated with reduced synaptic plasticity in the hippocampus of mice (Lambert et al., 1998). In human AD brains, increasing soluble A β correlates with decreased synaptic density and soluble A β accounts of 40% of the variability in synaptic density (Lue et al., 1999). There are mechanisms by which A β and NFTs can both contribute to the neuronal and synaptic loss observed in AD.

1.2.1.4 Astrocytes

Astrocytes are brain cells that are essential for maintaining homeostasis and ensuring the proper functioning of neurons. Vital functions of astrocytes include providing nutrients, growth factors and metabolites to neurons, maintaining BBB integrity, supporting anaerobic metabolism, antioxidant function and responding to inflammation (Steele & Robinson, 2012). Astrocytes become reactive in response to tissue injury. This involves alterations to their morphology and changes in their primary functions. Reactive astrocytes become hypertrophic, showing an enlarged soma and thicker processes compared to healthy astrocytes; there are also changes in the expression of certain astrocyte-specific proteins (Sofroniew, 2009; Wilhelmsson et al., 2006). It has been recently shown that there is no typical phenotype of reactive astrocytes and the changes in protein expression are heterogeneous, depending on the type of injury sustained (Anderson, Ao, & Sofroniew, 2014; Zamanian et al., 2012). In *post-mortem* AD tissue there is an increase in expression of glial fibrillary acidic protein (GFAP) (Schechter, Yen, & Terry, 1981), S100b (Griffin et al., 1989; Sheng et al., 1996) and a decrease in glutamine synthetase (GS) (Robinson, 2000, 2001). These changes indicate the presence of reactive astrogliosis. Reactive astrocytes may provide

neurons with less metabolic support and increase the chances of synaptic and neuronal degeneration (Steele & Robinson, 2012).

Whether reactive astrogliosis precedes or follows other neuropathological changes in AD is still under investigation (Rodríguez-Arellano, Parpura, Zorec, & Verkhratsky, 2016). Many researchers, including those who support the amyloid cascade hypothesis, state that A β is deposited first, followed by astrocyte activation in response to A β (Dewitt, Perry, Cohen, Doller, & Silver, 1998; Hardy & Selkoe, 2002). Recently, PET imaging was used to visualise astrogliosis in the brain of AD and MCI patients. A positive correlation was found between astrogliosis and A β deposition in AD patients, and a negative correlation was found between astrogliosis and grey matter density in MCI patients (I. H. Choo, Carter, Schöll, & Nordberg, 2014). As no correlation was found between A β deposition and astrogliosis in MCI patients, these results suggest that astrogliosis may have different effects at different disease stages. This group has also found a decrease in astrogliosis over time (mean follow up time 2.8 years) in FAD patients (Rodríguez-Vieitez et al., 2016). Interestingly, the lower level of astrogliosis coincided with the appearance of clinical AD symptoms. Prolonged astrogliosis may impact on the level of support provided to neurons, which in turn would increase the risk of neuronal death and promote disease progression. In later stages of AD, reactive astrocytes are frequently associated with A β plaques (Nagele, D'Andrea, Lee, Venkataraman, & Wang, 2003; Robinson, 2000, 2001). The finding that astrocytes are able to degrade A β *in vitro* (Wyss-Coray et al., 2003) might explain why they co-occur in such proximity.

1.2.1.5 Microglia

Microglial cells are the primary immune response cells of the brain. They activate and mount an inflammatory response to brain injury (Aloisi, 2001). This inflammatory response includes an increase in the number of microglial cells present in the affected area, as well as the release of numerous cytokines (Block & Hong, 2005). In AD, there is an increased number of microglia, particularly in the hippocampus (Rodríguez et al., 2013) and in *post-mortem* tissue they are found to be associated with both A β plaques (Dickson et al., 1988) and NFTs (Sheng, Mrak, & Griffin, 1997). Further, there are increases in the expression of inflammatory cytokines, including interleukin (IL)-1 (Griffin, Sheng, Roberts, & Mrak, 1995; Griffin et al., 1989) and tumor necrosis factor alpha (TNF- α) (Tarkowski, Blennow, Wallin, & Tarkowski, 1999). Similarly to astrocytes there is debate regarding the order of pathological events (Calsolaro & Edison, 2016); are microglial cells being activated by the

presence of A β plaques and NFTs or are they responding to an unknown causative factor of AD? Of course there are multiple pathways of inflammatory activation and it is likely that once the neurodegeneration process begins proinflammatory pathways are continually being activated.

1.2.1.6 Oligodendrocytes and white matter

Oligodendrocytes are the glial cells responsible for producing and maintaining the myelin that ensheaths axons in the brain. Myelin sheaths are essential for fast and effective neural transmission and they are the distinguishing feature of white matter (Bradl & Lassmann, 2010). In ageing, it has been found that there is gradual deterioration of white matter from middle age onwards (Bartzokis et al., 2001; Ge et al., 2002a, 2002b). Bartzokis and colleagues (2001) reported an inverse U relationship between MRI white matter volume and age, with the peak white matter volume occurring at 45 and 48 years of age in the frontal and temporal lobes respectively. Further, a negative correlation was found between age and the global number of neocortical oligodendrocytes in males, but not in females (Pelvig, Pakkenberg, Stark, & Pakkenberg, 2008).

The extent of reductions in white matter volume seen in AD and MCI are greater than that observed in normal ageing (Alves et al., 2012; Ihara et al., 2010). This reduction has been shown to be equally due to the loss of myelin, oligodendrocytes and axons (Englund & Brun, 1990; Sjöbeck & Englund, 2003). A histology study reported a significant decrease in myelin in the frontal lobe of AD brains compared to controls, and a 3-fold increase in the percentage of degraded myelin, but did not find any changes in the temporal lobe (Ihara et al., 2010). Imaging studies have found damage in functional white matter tracts in both MCI and AD; in AD, damage was present in all major white matter tracts whereas in MCI the injury was less wide spread (Agosta et al., 2011; Alves et al., 2012; Pini et al., 2016). Further, discrete imaging measurements have been developed that are believed to be representative of axonal damage and myelin degradation. Such studies suggest that axonal damage is present in the corpus callosum, uncinate fasciculus, inferior and superior longitudinal fasciculi and inferior fronto-occipital fasciculus of AD patients. Myelin degradation is seen in the fornix, inferior and superior longitudinal fasciculi, inferior fronto-occipital fasciculus, corpus callosum and the uncinate fasciculus of AD patients (Agosta et al., 2011; Pievani et al., 2010). The uncinate fasciculus is particularly interesting as it has connections to many of the areas where early neuropathological changes are seen in AD, including the hippocampus, pyriform cortex and

parts of the frontal cortex, and it is functionally implicated in episodic memory (Von Der Heide, Skipper, Klobusicky, & Olson, 2013).

It has been suggested that the cessation of myelin production in the brain around midlife may be the trigger for the development of AD (Bartzokis, 2004). This theory suggests that when myelination stops in midlife, demyelination begins in the reverse order; with axons that are myelinated late in development undergoing demyelination before those axons that were myelinated earlier in development. Bartzokis (2011) proposed that this demyelination process triggers the accumulation of A β and NFTs. Animal studies seem to support this conjecture, with myelin damage being evident prior to the accumulation of A β and NFTs in transgenic mice (Desai et al., 2009). However it has also been shown that A β can cause significant toxicity to oligodendrocyte cell cultures (Desai et al., 2010). Bartzokis, Lu, and Mintz (2007) suggest that demyelination leads to the oligomerisation of A β and the release of iron from oligodendrocytes. The A β and iron then form the classical AD plaques, of which iron is a key component. It may be that the initial demyelination leads to A β deposition, which then perpetuates a cycle of further myelin damage, and A β deposition.

1.2.1.7 Other neuropathological changes in AD

There are other neuropathological changes associated with AD that have not been mentioned thus far, and as they do not form a key part of this thesis they will not be discussed in detail. These changes include BBB leakiness (Marques, Sousa, Sousa, & Palha, 2013), alterations of blood vessels (Kalaria, 1996) as well as metal ion imbalances (Lovell, Robertson, Teesdale, Campbell, & Markesbery, 1998).

BBB leakiness could be reflective of the increase in reactive astrocytes present in the AD brain, as discussed above. These reactive astrocytes are less efficient in their role of maintaining BBB integrity therefore leading to leakiness (Obermeier, Daneman, & Ransohoff, 2013). In AD, decreased clearance of A β from the brain has been linked to BBB deficits (Mawuenyega et al., 2010).

There is evidence for the involvement of metal ions in AD (Bush, 2003; Huang, Moir, Tanzi, Bush, & Rogers, 2004), specifically iron (Peters, Connor, & Meadowcroft, 2015), copper (X. Y. Choo, Alukaidey, White, & Grubman, 2013) and zinc (Kulikova, Makarov, & Kozin, 2015). Iron and copper have the capacity to induce oxidative stress via redox cycling (Halliwell & Gutteridge, 2007), while zinc causes oxidative stress by inhibiting antioxidant

pathways within cells (Bishop, Dringen, & Robinson, 2007). The concentrations of iron, copper and zinc increase in AD, particularly within A β plaques (Lovell et al., 1998). A β may have a neuroprotective role in sequestering these metal ions in order to prevent oxidative stress in neurons (Bishop & Robinson, 2004). The mechanisms by which this might occur are yet to be identified. Since a large amount of iron is contained in myelin, if myelin degradation is the initial pathological feature of AD (Bartzokis, 2011), the release of iron from myelin may initiate iron dysregulation which has been hypothesised to trigger the production of A β in order to sequester the free iron and prevent toxicity (Bishop et al., 2002; Peters et al., 2015).

1.2.1.8 AD pathology in the brainstem

As well as cortical pathology, there is also subcortical neuropathology in AD, particularly in the brainstem. AD patients have been found to have reduced total brainstem volume compared to healthy controls, as measured by MRI (J. H. Lee, Ryan, Andreescu, Aizenstein, & Lim, 2015). Further, while specific regional differences in the brainstem could not be determined, it was noted that the posterior portion of the brainstem had more structural abnormalities in AD patients than in controls (J. H. Lee, Jung, Choi, & Lim, 2016; J. H. Lee et al., 2015). Neuropathological studies have shown that NFTs and A β plaques are present in the brainstem in AD, with higher densities being observed in the posterior regions adjacent to the fourth ventricle and cerebral aqueduct (Iseki et al., 1989; Parvizi, Van Hoesen, & Damasio, 2001). Specific brainstem nuclei have been investigated for accumulation of both NFTs and A β plaques. Giess and Schlote (1995) investigated 10 AD brains and found NFTs in all brains in the reticular formation at the level of the medulla, central superior nucleus in the pons, dorsal raphe nucleus, periaqueductal grey, locus ceruleus (LC), parabrachial nucleus, pigmented subpeduncular nucleus, inferior colliculus and substantia nigra. A β plaques were found in all brains in the periaqueductal grey, inferior colliculus, parabrachial nucleus, LC, dorsal raphe nucleus and nucleus cuneiformis. Parvizi et al. (2001) examined 32 AD brains and found NFTs in all brains only in the rostral raphe complex which includes the dorsal raphe nucleus, and found A β plaques in all brains only in the inferior colliculus. Another study examined only the LC and found that AD brains had significantly more NFTs than control brains (Dugger et al., 2012). It has been suggested that NFT accumulation may begin in the brainstem in AD rather than in the entorhinal cortex (Braak & Del Tredici, 2015; Braak et al., 2011; Grinberg et al., 2009).

Neuronal loss has also been detected in AD in both the LC and dorsal raphe nucleus (C. P. L. H. Chen et al., 2000; Zarow, Lyness, Mortimer, & Chui, 2003). C. P. L. H. Chen et al. (2000) reported a mean neuronal loss of 41% in the dorsal raphe nucleus of AD patients compared to healthy controls, while Zarow et al. (2003) concluded that there is a 68% loss of neurons in the LC compared to controls. The latter study also reported high variability in the AD group with 25% reporting LC cell counts within the range of healthy controls (Zarow et al., 2003). Interestingly, these two nuclei have functions associated with the sleep/wake cycle and may be involved in the sleep disruptions seen in AD.

1.2.2 Risk factors for AD

There are many risk factors for AD, most of them are environmental and very few are genetic. The main genetic risk factor, apolipoprotein E will be discussed below. As for the non-genetic risk factors age is the most significant. Other risk factors include smoking, diabetes, physical inactivity and a history of head injury (Jiang, Yu, Tian, & Tan, 2013).

1.2.2.1 Apolipoprotein E

In sporadic AD, the most substantial genetic risk factor by far is the apolipoprotein E (Apo E) gene. The three alleles of the Apo E gene are called E2, E3 and E4; and since each gene is a pair there are six possible combinations of Apo E alleles; E2/E2, E2/E3, E2/E4, E3/E3, E3/E4 and E4/E4. The most common genotype is E3/E3 (Mahley, 1988). These genes encode for the Apo E protein which functions in cholesterol transport, injury repair and antioxidant defense (J. Kim et al., 2009; Mahley, 1988).

The E4 allele has been associated with an increased risk of developing AD. People with one or two E4 alleles have a 2- and 8-fold increased risk of AD, respectively (Corder et al., 1993). Interestingly, the E2 allele has been associated with a reduced risk of AD (Suri, Heise, Trachtenberg, & Mackay, 2013). Multiple reasons for the increased risk of AD in E4 allele carriers have been suggested (J. Kim et al., 2009), including a reduced capacity for neural repair (Mahley & Rall Jr, 2000), reduced antioxidant capacity (Ramassamy et al., 1999; Shea, Rogers, Ashline, Ortiz, & Sheu, 2002), a decreased ability to prevent tau phosphorylation (Strittmatter et al., 1994) and a decreased ability of Apo E4 to clear A β from the brain (Deane et al., 2008). Strittmatter et al. (1994) showed that Apo E3 but not Apo E4 can bind to tau proteins at particular sites. When unbound, these sites can lead to the formation of paired helical filaments which go on to form NFTs. Studies have found that Apo E4 knock-in mice

have increased levels of phosphorylated tau and A β compared to Apo E3 knock-in mice (Liraz, Boehm-Cagan, & Michaelson, 2013). A study in human subjects has found a significant relationship between the presence of the Apo E4 allele and NFT accumulation (A. S. P. Lim, Yu, et al., 2013). Moreover, this relationship was found to be modified by sleep consolidation, better sleep consolidation attenuated the effect of Apo E4 on NFT accumulation. Sleep consolidation had no effect on A β plaque deposition, even though there was increased A β pathology in Apo E4 allele carriers compared to non-carriers.

The exact mechanism by which Apo E and A β interact is not fully understood. However, there is evidence to suggest that Apo E plays a significant role in the clearance of A β from the brain. A β -Apo E4 complexes are cleared from the brains of mice at a significantly slower rate than A β -Apo E3 or A β -Apo E2 complexes (Deane et al., 2008). It has been confirmed that this effect is due to a decrease in the rate of clearance of A β rather than to an increase in the synthesis of A β (Castellano et al., 2011). It has been suggested that Apo E may also aid in the degradation of A β , however it is not known if this occurs in an allele dependent manner (J. Kim et al., 2009).

1.2.3 Preclinical AD

Neuropathological studies have found significant A β and NFT pathology in the brains of people without clinical dementia. One study found that all 26 cognitively normal participants examined over 75 years of age had some degree of NFT accumulation and almost 70% had A β plaque deposition in their brains (Price & Morris, 1999). This pattern suggests a preclinical phase of AD in which pathological changes occur before a threshold of damage is reached and clinical symptoms become noticeable. The content of CSF or blood has been used to identify biomarkers for AD. Generally, plasma A β 42 is decreased in AD patients compared to controls (Schneider, Hampel, & Buerger, 2009). It has been found that A β 42 levels decrease in CSF as severity increases (most commonly measured as conversion from MCI to AD), whereas phosphorylated tau levels in CSF increase with increasing severity (Diniz, Pinto Jr, & Forlenza, 2008). It is presumed that the CSF decrease in A β and the increase in tau correspond to accumulation of pathology in the brain. Reduced clearance of A β leads to the deposition of insoluble A β within the brain, and a consequent decrease in A β levels in CSF and blood. In contrast, it is thought that the secretion of phosphorylated tau and the gradual degradation of NFTs lead to an increase in soluble tau that results in elevated CSF levels (Hall & Saman, 2012).

A recent Cochrane review has found that neither CSF nor plasma A β levels are specific enough to be used clinically as biomarkers of MCI to AD conversion (C. Ritchie et al., 2014). A meta-analysis estimated the prevalence of preclinical amyloid deposition using data from both CSF and A β imaging studies (Jansen et al., 2015). They found that the prevalence of preclinical amyloid deposition increased with age from 10% at age 50 to 44% at age 90. The presence of A β in cognitively normal participants is associated with a decline in performance on working memory and learning tasks after 36 months compared to those without A β (Y. Y. Lim et al., 2014). PET imaging has led to estimates that A β accumulation may begin in the brain 30 years before AD is diagnosed (Villemagne et al., 2013), which suggests that there may be a very large window of time in which to intervene to prevent A β accumulation. In this context, it should be noted that an increase in CSF A β 42 has been seen in cognitively normal men after one night of sleep deprivation (Ooms et al., 2014), suggesting that disrupted sleep can affect the production and clearance of A β from the brain.

1.3 Obstructive Sleep Apnoea

Obstructive sleep apnoea (OSA) is a sleep disorder characterised by the collapse of the upper airway during sleep causing a disruption in air flow to the lungs. These cessations in breathing cause arousals from sleep in order to force the airway open and allow oxygen to flow again. A person with sleep apnoea may experience hundreds of these apnoeic events each night. This is particularly disruptive to the organ that consumes 20% of the body's oxygen, the brain. It is not surprising that sufferers of OSA often experience daytime sleepiness, attention lapses and reduced cognitive functioning (Aloia, Arnedt, Davis, Riggs, & Byrd, 2004). The prevalence of OSA has been estimated in various studies, and while it is reported that OSA affects approximately 7-10% of the general population (Punjabi, 2008; Young et al., 1993), a recent review has suggested the prevalence is much higher, affecting 9-38% of the adult population (Senaratna et al., 2017), and it is likely that a large proportion remain undiagnosed (Simpson et al., 2013; Singh et al., 2013).

There are many risk factors for the development of OSA, including: i) age: OSA affects less than 10 % of people aged 30-39 years and close to 50 % of people aged 60-70 (Durán, Esnaola, Rubio, & Iztueta, 2001); ii) gender: Males have at least 2:1 increased odds of having OSA compared to females (Punjabi, 2008). This is reportedly due to sex hormone differences, with pre-menopausal women and post-menopausal women on hormone

replacement therapy having a lower prevalence of OSA than post-menopausal women who are not on hormone replacement therapy (Bixler et al., 2001). iii) Body mass index (BMI): people who have BMI rating of 30 or above are classed as obese and are more likely to develop OSA (Durán et al., 2001; Gislason, 2014). This has been associated with changes in the structure and function of the upper airway as well as increased neck circumference (Mortimore, Marshall, Wraith, Sellar, & Douglas, 1998; Strobel & Rosen, 1996). Additionally, people with obesity have an increased risk of cardiovascular disease and diabetes, both of which are found to be associated with OSA (Gislason, 2014). There may be an interaction occurring between multiple morbidities to increase susceptibility to OSA.

OSA is often diagnosed after being noticed by the patient's bed partner, suggesting that OSA patients themselves are unaware of the apnoeic episodes and many people are likely to remain undiagnosed for this reason (Punjabi, 2008). Consequently it is often not known how long a patient has suffered with OSA before being diagnosed. OSA is associated with an increased risk of motor vehicle accidents and workplace accidents. A meta-analysis found that people with OSA have 2.43 times increased risk of a car accident than controls (Tregear, Reston, Schoelles, & Phillips, 2009). This risk was found to be affected by disease severity, BMI and daytime sleepiness. As well, OSA is associated with lifelong increased risks for cardiovascular disease, stroke, diabetes and depression (Gislason, 2014; Gupta & Simpson, 2015). However, many of these conditions are common comorbidities of obesity and therefore the influence of OSA is difficult to isolate. OSA is also a risk factor for the development of MCI and dementia in old age (discussed in detail below) (Yaffe et al., 2011).

1.3.1 Classification and Treatment of OSA

OSA is diagnosed by polysomnography (PSG) during an overnight sleep study. The PSG records the number of apnoeas and hypopnoeas during sleep as well as the percentage of arterial oxygen desaturation and a number of other sleep variables. OSA is categorised by severity according to the apnoea/hypopnoea index (AHI) or oxygen desaturation index (ODI). The AHI is a measure of the number of apnoeas (complete collapse of the airway), and/or hypopnoeas (partial collapse of the airway), per hour of sleep (Gislason, 2014). The ODI is a measure of the number of arterial oxygen desaturations of greater than 3% or 4%, per hour of sleep (Ling, James, & Hillman, 2012). Scores of <5 events per hour on both the AHI and ODI are considered normal. Patients classified as having mild OSA have between 5 and 15 events per hour on the AHI. Moderate OSA is classified as 15-30 events per hour and severe OSA is

>30 events per hour on the AHI (Gislason, 2014). While AHI is the most common measure used to diagnose OSA, it is very strongly correlated with ODI values (Ernst et al., 2016).

Due to the frequent arousals during the night, OSA patients experience changes in sleep architecture compared to normal sleep patterns. Normally, stage 1 NREM sleep accounts for 3-8% of total sleep time, stage 2 comprises 45-55% of total sleep time, stages 3 and 4 (also known as slow wave sleep) account for 15-20% of total sleep time. REM sleep accounts for 20-25% of sleep time (Rama et al., 2005). Patients with OSA experience significantly less restful stage 3 and 4 sleep and more light stage 1 and 2 sleep (H. Kim et al., 2016; Yaouhi et al., 2009). Yaouhi et al. (2009) found that OSA patients spent an average of 54% of sleep time in stage 1 & 2, 19% in stage 3 and 4 and 15% in REM sleep. These values are similar to those seen in people without sleep disorders (above) and the percentage of time spent in each of these sleep stages amounts to 88% of sleep time, the study fails to account for the remaining 12%. Other studies have found the percentage of sleep time spent in stage 1 and 2 sleep in OSA patients to be 70-85%, stage 3 and 4 to be 3-10% and REM sleep to be 5-23% (Joyeux-Faure et al., 2016; H. Kim et al., 2016; S. J. Kim, Lee, Lee, Jhoo, & Woo, 2011; Kushida et al., 2012; Roure et al., 2008). Most of these studies did not have control groups, however when compared to the above percentage values from the healthy adult population these figures suggest that OSA patients spend more time in light sleep stages (1 and 2) and less time in deep sleep (stages 3 and 4). The two studies that did compare OSA patients to healthy controls reported that OSA patients spend significantly more time in stage 1 and 2 sleep and significantly less time in stage 3 and 4 sleep (H. Kim et al., 2016; Yaouhi et al., 2009). H. Kim et al. (2016) also found OSA patients have significantly less total sleep time and significantly less REM sleep compared to controls. However, the control group in this study was found to be significantly younger (mean age 44 vs 50 years) than the patient group, and as total sleep time and time spent in REM sleep decrease with age (Ohayon et al., 2004) these results should be interpreted with caution. Regardless of these considerations, it is clear that OSA patients do experience significant changes in sleep architecture.

The most commonly used treatment for OSA is continuous positive airway pressure (CPAP) which is a mask worn during sleep to ensure a constant flow of air to the lungs. The use of CPAP has been found to reduce the number of apnoeas and hypopnoeas measured by the AHI, and the time spent with arterial oxygen saturation below 90% to within normal ranges (Sullivan, Berthon-Jones, Issa, & Eves, 1981). It also normalises the sleep patterns of OSA patients (Sullivan et al., 1981). The percentage of time spent in stage 1 and 2 was decreased

from 73% to 42% after CPAP treatment, stage 3 and 4 increased from 5% to 25% and REM sleep increased from 22% to 33% after treatment (Sullivan et al., 1981). While CPAP is an effective treatment for the symptoms of OSA, particularly excessive daytime sleepiness (Rajagopal, Bennett, Dillard, Tellis, & Tenholder, 1986), there are compliance issues as it can be uncomfortable to use (Weaver & Grunstein, 2008). Therefore not all diagnosed patients receive the benefits of CPAP treatment.

1.3.2 OSA as a risk factor for AD

As mentioned in section 1.1.4 above, there is an increased prevalence of OSA/SDB in people with AD/dementia. Indeed it has been found by a recent meta-analysis that people with AD have a 5.05-fold increased risk of having OSA (Emamian et al., 2016). Since OSA is typically diagnosed in midlife, decades before signs of AD/dementia are seen, this suggests that OSA is a risk factor for AD. While this idea has been proposed frequently (Daulatzai, 2012, 2013; Pan & Kastin, 2014), there is currently limited evidence to back up the claim (Baumgart et al., 2015). However, the presence of SDB or OSA has been found to be an independent risk factor for the development of MCI or dementia (Chang et al., 2013; Yaffe et al., 2011). Patients with OSA have a 1.70-fold (Chang et al., 2013) or 1.85-fold (Yaffe et al., 2011) increased risk of developing dementia within the next 5 year period. Further, Yaffe (2011) found that an ODI ≥ 15 (equivalent to moderate OSA) and longer time spent in apnoea/hypopnoea were both associated with this increased risk. In this study, sleep fragmentation and total sleep time were not associated with an increased risk of dementia, suggesting that hypoxia rather than sleep fragmentation is responsible for the increased risk. Conversely, a study has found that a higher rate of sleep fragmentation in non-demented elderly people is associated with faster cognitive decline and an increased risk of AD compared to those with lower sleep fragmentation rates (A. S. P. Lim, Kowgier, Yu, Buchman, & Bennett, 2013). However, the measurement of sleep fragmentation in this study was taken from actigraphy watches, which measure arousals during sleep; neither PSG nor arterial oxygen desaturation were measured. Since it is possible that the sleep fragmentation observed was attributable to OSA, this study does not provide reliable support for the notion that sleep fragmentation is an independent risk factor for AD.

Treatment of OSA or SDB in AD/dementia improves sleep quality and slows the progression of cognitive decline (Cooke, Ancoli-Israel, et al., 2009; Cooke, Ayalon, et al., 2009; Troussière et al., 2014). Three weeks of CPAP treatment significantly decreased the

percentage time spent in stage 1 sleep and increased the time spent in stages 2 and 3 in AD patients with OSA (Cooke, Ancoli-Israel, et al., 2009). Moreover, after three weeks of CPAP treatment AD patients with OSA report significant improvements in subjective daytime sleepiness (Chong et al., 2006) and cognition (Ancoli-Israel et al., 2008). The benefits of regular CPAP treatment for reducing the cognitive symptoms in early dementia persist for 1-2 years, as patients exhibit significantly slower cognitive decline compared to non-CPAP users (Cooke, Ayalon, et al., 2009; Troussière et al., 2014). This evidence that CPAP benefits both OSA and AD/dementia strengthens the likelihood that both disease states are causally linked.

Interestingly, treatments for AD have also been shown to improve OSA symptoms. Donepezil, an acetylcholinesterase inhibitor, is commonly prescribed to slow the cognitive decline in AD. A sleep study in AD patients found that those receiving Donepezil had 10 % less stage 1 sleep and 10 % more stage 2 sleep compared to AD patients not taking the drug (Cooke et al., 2006). However, the mean AHI values for the groups were not specified in this study, although it was stated that there were no differences between groups on the AHI. Another study found that AD patients with OSA who were treated with Donepezil for one month experienced significantly more REM sleep, lower AHI values and improved cognition when compared to placebo-treated controls (Moraes, Poyares, Sukys-Claudino, Guilleminault, & Tufik, 2008). Additionally, a group of OSA patients without AD exhibited improvements in AHI values, oxygenation levels and subjective sleepiness after one month of treatment with Donepezil, when compared to placebo-treated controls (Sukys-Claudino, Moraes, Guilleminault, Tufik, & Poyares, 2012). The basis of this beneficial effect of Donepezil on OSA severity is not yet known; perhaps it is related to an increase in the tone of the pharyngeal musculature.

The improved cognitive performance in patients with OSA who receive Donepezil may be a direct result of the reduced OSA symptoms and better sleep. However an additional mechanism has been proposed. It has been suggested that acetylcholinesterase inhibitors may have additional non-synaptic effects that improve myelin integrity (Bartzokis, 2007). In support of this idea, recent cell culture studies have found increased oligodendrocyte differentiation and an increased expression of myelin-related proteins after treatment with Donepezil (Imamura et al., 2015; Imamura, Arai, Dateki, & Takishima, 2017). Decreases in white matter integrity are seen in both AD (discussed above) and OSA (to be discussed

below), thus some of Donepezil's beneficial effects on cognition may be attributable to the repair or prevention of myelin damage.

1.3.3 The two types of damage in OSA

Two factors have been identified that contribute to the symptoms in OSA: sleep fragmentation and intermittent hypoxia. Sleep fragmentation occurs due to the numerous arousals and the insufficient slow wave sleep experienced by OSA patients. Human sleep fragmentation studies use auditory or mechanical stimuli to cause sleep fragmentation, and to ensure that the total sleep time is not reduced. Such studies have found decreases in objective and subjective sleepiness, psychomotor vigilance, attention and some executive functions (Bonnet & Arand, 2003; K. Jones & Harrison, 2001; Ko, Fang, Tsai, & Hsieh, 2015). Such results demonstrate that altered sleep architecture is sufficient by itself to cause a pattern of cognitive impairments that is similar to that seen in OSA (discussed further below). It would be interesting to know whether sleep fragmentation is associated with an increase in the cerebral burden of A β or NFTs.

During the course of sleep, the apnoeic episodes in OSA can lead to a progressive worsening of arterial oxygen saturation, from the normal range of 98-100% down to levels as low as 50-90%; these low levels place the brain under considerable metabolic stress. Studies investigating the effects of high altitudes in humans can be useful in determining the effects of hypoxia without sleep fragmentation. Such studies have shown deficits in mood and cognitive tasks, including attention, visual and working memory, speed of processing and concentration (De Aquino Lemos et al., 2012). Serum increases in inflammatory cytokines have also been found in participants exposed to high altitudes (Hartmann et al., 2000). High altitude exposure has been associated with alterations in sleep, breathing and increased AHI scores resulting in fragmentation of sleep (De Aquino Lemos et al., 2012; Latshang et al., 2013). These effects mean that high altitude studies are not ideal for assessing the contribution of hypoxia to OSA, as the unique contribution of hypoxia cannot be determined. A recent study has subjected participants directly to hypoxic gas and found significant deficits in memory, speed of processing, executive functioning, attention and cognitive flexibility (Turner, Barker-Collo, Connell, & Gant, 2015). However, this method and exposure to high altitude results in continuous hypoxia, which the body adjusts to over time, whereas the intermittent hypoxia in OSA does not appear to lead to a compensatory response.

1.3.4 OSA and cognition

OSA is associated with significant cognitive deficits; however, the cognitive domains that have been reported to be affected vary between studies. People with OSA have been reported to perform poorly on neuropsychological tests that measure attention/vigilance, both short- and long-term memory, motor performance, visuospatial learning, constructional ability and executive functioning (Beebe & Gozal, 2002; Ferini-Strambi et al., 2003). Furthermore, daytime sleepiness and depressive symptoms are both increased in OSA (El-Sherbini, Bediwy, & El-Mitwalli, 2011; Yaouhi et al., 2009). A meta-review analysing five meta-analyses/systematic reviews of OSA and cognitive deficits concluded that there was support for deficits in attention/vigilance, visual and verbal memory, executive functions and constructional abilities, with sparing of language and psychomotor skills (Bucks, Olaithe, & Eastwood, 2013). These conclusions may be biased because there was considerable overlap with the original studies used in the five meta-analyses/systematic reviews investigated. For instance, data from one original paper was used in all five reviews, possibly skewing the results of this meta-review. On the other hand, a recent study has reported deficits in attention, verbal, visual and working memory, executive functioning and general cognition in OSA patients compared to healthy controls, with no difference on tests of language (Rosenzweig et al., 2016). It is notable that there is significant overlap between these deficits in OSA and the cognitive deficits seen in AD, although in AD deficits are more severe and not reversible with treatment (Burns & Iliffe, 2009).

Studies that have investigated the effectiveness of CPAP treatment in reversing the cognitive deficits in OSA have obtained inconsistent results (Kylstra, Aaronson, Hofman, & Schmand, 2013). The cognitive symptoms of OSA that are usually seen to improve with CPAP treatment include memory, visuospatial learning, motor performance, vigilance and attention (Canessa et al., 2011; Cohen-Zion et al., 2001; Ferini-Strambi et al., 2003). Executive functioning is one area of cognition that seems not to respond to treatment (Lau, Eskes, Morrison, Rajda, & Spurr, 2010; Saunamäki & Jehkonen, 2007). In particular, OSA patients perform worse than healthy controls at working memory tasks when measured as an aspect of executive functioning, and treatment with CPAP seems ineffective (Adams, Strauss, Schluchter, & Redline, 2001; Torelli et al., 2011). Halbower (2006) found that even children with OSA perform worse than age-matched controls in working memory tasks. It has been suggested that the reason treatment is ineffective for working memory difficulties is that the deficit is caused by the hypoxia aspect of OSA, rather than by the sleep fragmentation aspect (Ferini-Strambi et al., 2003). The sleep fragmentation part of the disorder is alleviated with

CPAP treatment, as evidenced by normalised sleep patterns and lack of apnoeic events, while the intermittent hypoxia may have caused irreversible damage to areas of the brain that are associated with memory and executive function. In support of this conclusion, a recent study found no differences in performance between OSA patients treated with CPAP vs sham CPAP for one month, on tests of episodic, procedural or working memory (Joyeux-Faure et al., 2016). While these results support the idea that working memory deficits in OSA cannot be reversed by CPAP, they also show that other types of memory deficits are also irreversible.

1.3.5 OSA and structural brain changes

As well as cognitive deficits, many structural changes have been found in the brains of patients with OSA, including volumetric loss, reduced activation and metabolite changes.

1.3.5.1 OSA and structural grey matter abnormalities

MRI studies have compared the regional volumes of grey matter in OSA patients to that in healthy controls. Grey matter loss has been consistently seen in the hippocampus, parahippocampus and temporal lobes of OSA patients (Canessa et al., 2011; Gale & Hopkins, 2004; Macey et al., 2002; Morrell et al., 2003; Torelli et al., 2011; Yaouhi et al., 2009). Indeed, a recent meta-analysis of MRI studies in OSA patients found reduced grey matter volume in the parahippocampus bilaterally (Weng et al., 2014). Further, Canessa et al. (2011) reported a negative correlation between AHI and grey matter volume in the hippocampus. Less consistent decreases in grey matter volume in OSA patients have been found in frontal and parietal cortices, cingulate, cerebellum, mammillary bodies and some parts of the basal ganglia (Canessa et al., 2011; H. Kim et al., 2016; Kumar et al., 2008; Macey et al., 2002; Morrell et al., 2010; Torelli et al., 2011; Yaouhi et al., 2009). Grey matter volume loss in the hippocampus of OSA patients is correlated with deficits in verbal and visuospatial memory as well as in working memory and executive functioning (Canessa et al., 2011; Gale & Hopkins, 2004). However, some studies failed to find significant correlations between regions of grey matter loss and neuropsychological test scores (Torelli et al., 2011; Yaouhi et al., 2009), instead suggesting that neuropathological changes are likely to precede cognitive deficits in OSA.

Interestingly, many of the regions where grey matter volume is reduced in OSA are the same regions where grey matter volume is reduced in AD, specifically the hippocampus and

temporal lobe, frontal cortex, cingulate and basal ganglia regions (Baron et al., 2001; Frisoni et al., 2002; Karas et al., 2004). One difference is the cerebellum, which experiences grey matter loss in OSA (H. Kim et al., 2016; Macey et al., 2002; Morrell et al., 2010; Yaouhi et al., 2009) whereas data for AD are equivocal. Some studies have reported neuronal loss, gliosis and atrophy in the cerebellum in AD (Sjöbeck & Englund, 2001; Thomann et al., 2008), whereas other studies have found no significant volumetric differences between AD and controls (Mrzilková, Zach, Bartoš, Tintěra, & Řípková, 2012).

Two studies have investigated the effect of CPAP treatment on grey matter volume in OSA patients, and obtained similar findings (Canessa et al., 2011; H. Kim et al., 2016). Both studies reported increases in grey matter volume after CPAP treatment in the hippocampus, superior frontal and orbitofrontal cortices. Canessa et al. (2011) found additional volumetric increases in the parahippocampal gyrus, while H. Kim et al. (2016) found volumetric increases in the anterior cingulate, precuneus, prefrontal cortex and parts of parietal and temporal cortices. These differences may be due to the length of CPAP treatment: Canessa et al. (2011) used a three month CPAP intervention whereas H. Kim et al. (2016) had an average CPAP duration of 18 months with the minimum duration of eight months. This difference may explain the additional areas of increased volume in the latter study. H. Kim et al. (2016) also noted substantial overlap between areas of decreased volume before treatment and areas of increased volume after treatment, consistent with recovery of the damaged regions.

1.3.5.2 OSA and structural white matter abnormalities

Many studies have used diffusion tensor imaging (DTI) to investigate the white matter integrity of the brain in OSA. A number of imaging markers have been analysed as proxies for measuring different aspects of white matter tissue. Fractional anisotropy and magnetisation transfer ratio are used as general measures of white matter integrity, mean diffusivity and mean kurtosis are used to determine tissue injury with decreased diffusivity or increased kurtosis indicating acute white matter injury and increased diffusivity or decreased kurtosis indicating chronic injury (Kumar et al., 2012; Tummala, Palomares, et al., 2016). All studies that have investigated white matter integrity in OSA patients have reported lower white matter volumes compared to in healthy controls, however the specific regions of loss vary between studies (Castronovo et al., 2014; H. L. Chen et al., 2015; Macey et al., 2008; Tummala, Roy, et al., 2016; Xiong et al., 2016). Some common regions of decreased white

matter integrity include sections of the superior longitudinal fasciculus, cingulum bundle, corpus callosum and temporal regions (Castronovo et al., 2014; H. L. Chen et al., 2015; Macey et al., 2008; Tummala, Roy, et al., 2016). Other areas that have been implicated less frequently include inferior fronto-occipital fasciculus, internal and external capsule, prefrontal and parietal regions (H. L. Chen et al., 2015; Macey et al., 2008; Tummala, Roy, et al., 2016). Most studies have found acute tissue injury (decreased mean diffusivity or increased mean kurtosis). Similar to white matter integrity measures, there is overlap but little consistency between the regions in which tissue injury has been reported. Acute injury has been reported in at least two studies in the cerebellum, basal ganglia, corpus callosum, frontal cortex, hippocampus/temporal lobe, parietal cortex and limbic regions (Castronovo et al., 2014; Kumar et al., 2012; Tummala, Palomares, et al., 2016). However, another study found no mean diffusivity changes between OSA patients and controls in any regions (H. L. Chen et al., 2015). Interestingly, a study investigating mean diffusivity changes between OSA patients with residual sleepiness after CPAP treatment and those without residual sleepiness found increased mean diffusivity in the sleepy OSA patients (Xiong et al., 2016). This increase, indicative of chronic tissue injury, was seen in the corona radiata, corpus callosum and external capsule and implies that residual sleepiness is associated with long-lasting, possibly permanent tissue injury in OSA.

Only one study has investigated the effect of CPAP treatment on white matter integrity and damage in OSA patients (Castronovo et al., 2014). After three months of treatment, baseline decreases in fractional anisotropy and mean diffusivity in the superior longitudinal fasciculus were reversed as well as baseline decreased fractional anisotropy in the uncinate fasciculus. At 12 months follow up, the improvement persisted in the superior longitudinal fasciculus. Clearly, more research is necessary to better understand the pattern of white matter damage in OSA and whether treatment can attenuate this damage.

In contrast to the situation in OSA, white matter deterioration is seen across all regions of the brain in AD, with the deterioration being less severe in MCI (Agosta et al., 2011; Alves et al., 2012). This pattern suggests that white matter damage may begin during the pre-MCI stage and then progressively worsen as the disease progresses.

1.3.5.3 OSA and functional brain abnormalities

Functional MRI (fMRI) is used to examine the extent of regional brain activation while a subject is performing a cognitive task. Studies that have used fMRI to compare patients with

OSA to healthy controls have obtained inconsistent findings. One group of studies has found increased brain activation in OSA. For instance, Ayalon, Ancoli-Israel, Klemfuss, Shalauta, and Drummond (2006) conducted fMRI while subjects were performing a verbal learning task. Healthy controls and OSA patients performed equally well on the task, yet the OSA patients had increased activation in inferior and middle frontal gyri, cingulate gyrus, cerebellum, thalamus and the junction of the inferior parietal and superior temporal gyri. The authors suggested that this pattern indicates a compensatory mechanism whereby more brain regions need to be recruited in order to complete the task effectively. Similarly, Castronovo et al. (2009) found that OSA patients and controls performed equally well on a working memory task (the 2-back task), yet there was increased activation in the hippocampus and part of the frontal cortex in the OSA patients. Prilipko et al. (2011) also obtained comparable performance between controls and OSA patients on the 2-back task, and observed increased activation in temporal and occipital regions and decreased activation in precentral and middle frontal gyri.

In contrast, a second group of fMRI studies have reported decreased activation in OSA. Thomas, Rosen, Stern, Weiss, and Kwong (2005) reported that OSA patients performed worse than controls on the 2-back task and that this was accompanied by decreased activation in the dorso-lateral prefrontal cortex. Further studies have used the Go-NoGo task to test sustained attention and response inhibition in OSA patients compared to controls (Ayalon, Ancoli-Israel, Aka, McKenna, & Drummond, 2009; Ayalon, Ancoli-Israel, & Drummond, 2009). They found similar levels of sustained attention in the two groups but there was decreased activation in the cingulate, frontal and parietal regions of OSA patients. However, the OSA patients performed worse on the response inhibition part of the task compared to controls and decreased activation was found in the post central, cingulate, inferior parietal and insula cortices as well as the putamen.

Although these two groups of studies appear to have obtained opposing results with fMRI, it could be that the differences are due to baseline cognitive differences in the OSA patients. In the first group, the OSA patients were able to perform as well as the healthy controls on the cognitive tasks, while in the second group of studies, the OSA patients performed worse on the tasks than the healthy controls. Perhaps their worse performance was due to an inability to recruit other brain areas to assist with performing the task. In this context it is notable that the only comparable fMRI study of AD patients used the 1-back working memory task (H. K. Lim et al., 2008). As expected, the AD patients showed impaired performance on this task,

and like the OSA patients that showed impaired cognitive performance, they exhibited decreased brain activation, in this instance in the ventro-lateral prefrontal cortex, frontal pole, insula and premotor cortex.

Three fMRI studies have assessed the effect of CPAP treatment on brain activation; all used the 2-back working memory task, and all found no improvements on the task after CPAP treatment, when compared to baseline performance. Despite this lack of cognitive improvement, both increases and decreases were seen in brain activation, but these changes were not consistent between studies. Thomas et al. (2005) reported increased activation of posterior parietal regions, while Aloia et al. (2009) also found increased inferior parietal activation, but found decreased activation in the insula. Castronovo et al. (2009) reported decreased activation in the hippocampus and inferior frontal gyrus (regions that had previously seen increased activation) as well as decreased anterior cingulate activation. Perhaps more consistent changes in brain activation would have been found if the patient groups had displayed improvement in cognitive performance following CPAP treatment.

Resting state fMRI has been used to investigate the functional connectivity of brain networks while no specific task is being performed. The brain regions activated during a state of wakeful rest are termed the default mode network. Many regions of increased and decreased functional connectivity have been discovered in OSA patients compared to controls, including those that make up the default mode network (H. J. Li et al., 2015; Park et al., 2016; Peng et al., 2014; Santarnecchi et al., 2013; Q. Zhang et al., 2013). It is thought that regions of decreased functional connectivity represent functional deficits in OSA, potentially in the default mode network of the brain, while increases in resting state activity represent functional compensatory mechanisms (Q. Zhang et al., 2013). Dysfunction in the default mode network has also been seen in AD (Rombouts, Barkhof, Goekoop, Stam, & Scheltens, 2005).

A meta-analysis of fMRI studies has reported significant convergent activation in the amygdala and the hippocampus using both task-based and resting state fMRI studies (Tahmasian et al., 2016). However, the task-based studies included in the analysis assessed different cognitive domains and the resting state studies assessed the default mode network so the only meaning that can be taken from this result is that perhaps the amygdala and hippocampus are more vulnerable to damage in OSA and require increased activation, even in a resting state.

1.3.5.4 OSA and metabolite abnormalities

Magnetic resonance spectroscopy (MRS) is used to measure key metabolites in the brain including N-acetylaspartate, creatine, choline, glutamate/glutamine and myo-inositol. Changes in these metabolites are considered to be markers of cellular or metabolic change. Unlike the inconsistent findings obtained between fMRI studies, most MRS studies have been in good agreement regarding changes in brain metabolites in the brains of patients with OSA.

Decreased levels of N-acetylaspartate are thought to indicate neuronal and/or axonal injury, and decreases in N-acetylaspartate concentration or the ratio of N-acetylaspartate and other metabolites have been consistently seen in the frontal cortices of OSA patients (Alchanatis et al., 2004; Algin et al., 2012; Kamba, Suto, Ohta, Inoue, & Matsuda, 1997; O'Donoghue et al., 2012; Sarchielli et al., 2008). The parieto-occipital cortex, temporal grey matter and insula cortex also have decreased N-acetylaspartate levels compared to healthy controls (Sharma et al., 2010; Tonon et al., 2007; Yadav et al., 2014). A contradictory finding of increased N-acetylaspartate/creatine ratio has been reported in the hippocampus of OSA patients compared to controls (Bartlett et al., 2004) and in severe OSA compared to mild OSA patients (Alkan et al., 2013). However, both studies attributed this result to a decrease in creatine rather than to an increase in N-acetylaspartate. N-acetylaspartate levels have been found to correlate negatively with AHI (Sarchielli et al., 2008; Sharma et al., 2010) suggesting that the extent of neuronal/axonal injury increases with increasing OSA severity. It has been suggested that the decreased N-acetylaspartate, indicative of neuronal injury, may be linked to the cognitive deficits seen in OSA, particularly executive dysfunction, as this is often associated with damage to the frontal lobes (Alchanatis et al., 2004). However, the only study to correlate brain metabolite changes with cognitive function found poor correlations with no significant relationships (O'Donoghue et al., 2012).

Choline levels are regarded as an indicator of membrane metabolism, and both increases and decreases in choline have been reported in the brains of OSA patients. Increases in the choline/creatine ratio have been seen in the thalamus, putamen, hippocampus and temporal lobe (Algin et al., 2012; Alkan et al., 2013; Sarchielli et al., 2008) and increased choline concentrations in the occipital lobe were correlated with increased AHI (Sharma et al., 2010). Decreases in the choline/creatine ratio have been reported in the hippocampus and frontal white matter (Alchanatis et al., 2004; O'Donoghue et al., 2012). O'Donoghue et al. (2012)

suggested that the levels of choline may change dynamically with injury duration, with increased choline in acute injury stages indicating increased membrane metabolism, and decreased choline at chronic stages of injury indicating impaired membrane metabolism and apoptosis. This conjecture would account for the differences seen in different studies.

Glutamate/glutamine represents combined glutamate and glutamine levels, and increases in glutamate/glutamine can indicate glutamate excitotoxicity or increased gliosis.

Glutamate/glutamine levels in the temporal lobe of OSA patients were significantly correlated with the severity of AHI (Sharma et al., 2010). While this study also reported higher glutamate/glutamine concentrations in the frontal and temporal lobes of OSA patients, these trends did not reach statistical significance compared to controls. Significantly increased glutamate/glutamine levels have also been seen in the insula of patients with OSA (Macey et al., 2016).

Myo-inositol is considered to be a glial marker and increases in myo-inositol levels are indicative of reactive gliosis and/or myelin breakdown. Myo-inositol levels are increased in the frontal, temporal and insula cortices of OSA patients compared to controls (Sarchielli et al., 2008; Sharma et al., 2010; Yadav et al., 2014) and the levels correlated positively with AHI in the insula and occipital cortices (Sharma et al., 2010; Yadav et al., 2014).

Creatine reflects energy metabolites and is usually used as a reference peak for comparing other metabolite changes. One study attributed a decreased N-acetylaspartate/creatinine ratio to decreased creatine levels rather than increased N-acetylaspartate levels and even found that lower creatine levels correlated with worse cognitive performance on a vigilance task in OSA patients (Bartlett et al., 2004).

Taken together, the results from MRS studies clearly indicate that OSA injures the brain. The decreased N-acetylaspartate levels show that neurons are injured, while the increased glutamate/glutamine and myo-inositol indicate increased gliosis, possibly in response to excitotoxicity or axonal injury. The decreased levels of creatine and the fluctuating levels of choline point to perturbed cellular metabolism. While these changes are evidently correlated with increased OSA severity, it is not yet clear what contributions are made by sleep disruption and hypoxia. Interestingly, metabolite changes have not been reported to change significantly after CPAP treatment (O'Donoghue et al., 2012; Tonon et al., 2007), which may indicate that OSA leads to irreversible reductions in brain energy metabolism.

In AD there are also changes in brain metabolites, and these changes resemble those seen in OSA in several respects. For instance, the brains of AD patients consistently display increased myo-inositol and decreased N-acetylaspartate/creatine in the hippocampus, cingulate, frontal, temporal, parietal and occipital cortices (Graff-Radford & Kantarci, 2013; N. Zhang et al., 2014). The spatiotemporal extent of these changes is associated with disease severity; it reflects the spatial progression of NFT and A β plaques, and may precede cognitive symptoms (S. Q. Chen et al., 2012; Kantarci et al., 2000). Like in OSA, the brain concentrations of creatine decrease in AD, but unlike in OSA, glutamate/glutamine levels are also reduced (N. Zhang et al., 2014).

1.3.6 Neuropathology of OSA

The results of cognitive, MRI and MRS studies all point to the likelihood that OSA injures the brain, and that the extent of injury is related to the severity of OSA. However, these three methods of investigation are unable to reveal the actual cellular changes that occur in specific brain regions. To date, only one study has investigated *post-mortem* brain tissue from patients with OSA, and the findings from that study will be described in detail below. The bulk of our knowledge about cellular changes in OSA has been deduced from animal models. Two types of animal model are used: intermittent hypoxia and sleep fragmentation. Neuropathological research using each of these models is discussed separately below.

1.3.6.1 Neuropathology of intermittent hypoxia (animal models)

Animal models of OSA involving intermittent hypoxia are often termed intermittent hypoxia (IH) or chronic intermittent hypoxia (CIH). For the purpose of this review, the abbreviation IH will be used and the duration of IH will be specified. IH models involve keeping animals, usually rodents, in an enclosed chamber and regulating the percentage of oxygen in the air. Oxygen levels in the chamber are briefly reduced to induce hypoxia and then returned to normal. The change in oxygen is timed and occurs intermittently throughout the time spent asleep, to mimic the pattern of apneic episodes in OSA. Using variations of this model, many inferences have been made regarding what may be occurring in the brains of OSA patients. Interestingly, animals exposed to IH show learning and memory deficits (Goldbart et al., 2003; D. Gozal, Row, Kheirandish, et al., 2003; L. J. Kim et al., 2015) that are similar to those seen in transgenic AD mice (W. Zhang et al., 2011). Specifically, rodents exposed to 14 days of IH during sleep showed spatial learning deficits on the Morris water maze task (D. Gozal, Row, Kheirandish, et al., 2003) as well as short- and long-term memory deficits in the

novel object recognition task compared to animals kept in room air (L. J. Kim et al., 2015). Performance deficits in the Morris water maze are also observed in transgenic AD mice (W. Zhang et al., 2011). Further, memory deficits are usually the first cognitive symptom observed in patients with OSA or with MCI.

Many of the neuropathological changes observed in IH-exposed rodents are similar to those seen in AD. Animals exposed to IH have increased neuronal apoptosis in the cerebral cortex and hippocampus, particularly in the CA1 region of the hippocampus (Fung, Xi, Zhang, Sampogna, & Chase, 2012; D. Gozal, Daniel, & Dohanich, 2001; D. Gozal, Row, Gozal, et al., 2003). The hippocampus is particularly vulnerable to hypoxic injury, and within the hippocampus some regions are more susceptible than others. The CA2 and CA3 regions are more resistant to hypoxic damage whereas the CA1 and dentate gyrus of the hippocampus are more vulnerable (E. Gozal et al., 2002; Hung, Tipoe, Poon, Reiter, & Fung, 2008; Ignacak et al., 2009). Interestingly, the CA1 is the region of the hippocampus that displays the greatest extent of neuronal loss in AD (Krill et al., 2004). Neuronal apoptosis has also been seen in the medulla and pons of the brainstem in response to as little as 3 h IH, with no apoptotic neurons being seen in control animals (J. H. Zhang, Fung, Xi, Sampogna, & Chase, 2010). Specifically, wake-active catecholaminergic neurons of the LC and periaqueductal grey in the brainstem are depleted by up to 40% after long-term (6 months) exposure to IH (Zhu et al., 2007). Neuronal cell death in IH is mediated by oxidative stress, including increased reactive oxygen species, lipid peroxidation, protein and nucleic acid oxidation (Wang, Zhang, & Gozal, 2010; W. Xu et al., 2004). Interestingly, oxidative damage can be attenuated by physical activity in an IH model (D. Gozal, Nair, & Goldbart, 2010).

Additionally, increased reactive gliosis is seen in IH, specifically an upregulation of GFAP in the CA1 and an increase in S100b, as well as morphological changes that are indicative of reactive astrogliosis (Angelo et al., 2014; Aviles-Reyes et al., 2010; E. Gozal et al., 2002). Surprisingly, Aviles-Reyes et al. (2010) found that the extent of neuronal cell death reduced with increased IH exposure and it was suggested that the increase in reactive astrogliosis may have been protective against IH-induced neuronal death. However, the maximum duration of IH in this study was 10 days, which limits the applicability of this finding to OSA patients who may be exposed to intermittent hypoxia for several decades of life.

Other studies have found that neuroinflammation is evident in the hippocampus only after chronic IH exposure (6 or 24 weeks) and not after acute exposure (1 day) (Sapin et al., 2015).

Inflammatory markers, particularly microglial markers increase in the cortex and brainstem of IH exposed animals compared to controls (Sapin et al., 2015; Smith, Friedle, & Watters, 2013; Yang, Wang, Feng, Cao, & Chen, 2013). Cortical microglial inflammatory markers are increased after 14 days IH exposure not 1 or 3 days, whereas brainstem inflammatory markers are increased after as little as 1 day IH exposure (Smith et al., 2013).

Evidence from animal models of OSA suggests that IH may hasten the progression of AD-like pathology. Cholinergic neurons are significantly depleted in the basal forebrain and spatial memory is impaired after 14 days of IH exposure in rats (Row, Kheirandish, Cheng, Rowell, & Gozal, 2007). Basal forebrain cholinergic neurons are also significantly depleted in AD (Ferreira-Vieira et al., 2016). Interestingly, cholinergic activation is decreased in the dentate gyrus after 14 days of IH exposure (Hambrecht et al., 2007). Transgenic AD mice exposed to IH for 4 weeks have significantly more A β accumulation than animals exposed to normal air (Shiota et al., 2013). Furthermore, increases in the amount of phosphorylated tau are seen in the hippocampus after exposure to hypoxia (Fang et al., 2010; C. E. Zhang et al., 2014), however these studies used sustained hypoxia rather than IH, so their relevance to OSA is questionable. Fang et al. (2010) exposed neuronal cell cultures to 2, 4, 6 or 8 h sustained hypoxia and mice to 2 or 4 h of hypoxia and found increased phosphorylated tau at all time points compared to normoxic animals or cells. The model used by C. E. Zhang et al. (2014) more closely resembles chronic hypoxia of OSA by exposing animals to hypoxia for 6 h per day, 6 days a week for up to 8 weeks, but this paradigm did not involve intermittent periods of reoxygenation characteristic of IH in OSA. They did, however, find increased levels of phosphorylated tau, markers of oxidative stress and spatial memory deficits, all of which are evident in AD (see section 1.2 above). While the concentrations of the transition metals copper, iron and zinc are increased in AD brains, they were not found to be increased in a model of long-term IH, however the levels of cobalt were significantly increased (Veasey et al., 2013). Increased cobalt was localised to white matter regions with axonal damage and myelin deterioration also being evident. Five times as many axons showed degenerative changes in the corpus callosum after IH compared to controls and expression of myelin-associated proteins was decreased. It was suggested that the deterioration of myelin in IH led to an increase in vitamin b12 which assists in myelin repair; cobalt is an essential component of vitamin b12, therefore the levels of cobalt were increased (Veasey et al., 2013).

There are considerable similarities between the pathological changes in AD brains and IH animal models. It is conceivable that many of the neuropathological changes characteristic of

AD and seen in IH models are also seen in the brains of OSA patients. An interesting finding regarding the IH model is that despite no experimental manipulation of the sleep cycle of mice exposed to IH, Veasey et al. (2004) found that IH exposed mice had significantly longer total sleep time and reduced sleep latency. The authors speculated that this may be due to the oxidative injury of wakefulness-promoting nuclei in the brainstem. If correct, it may indicate that IH has the capacity to increase hypersomnolence, independently of sleep fragmentation.

1.3.6.2 Neuropathology of sleep fragmentation (animal models)

Fewer studies have investigated the neuropathological outcomes of sleep fragmentation, which in rodents usually involves a treadmill moving at set intervals (usually every 2 min) in order to fragment sleep while not reducing the total sleep time. Such models have been shown to result in cognitive deficits, particularly in the domains of spatial memory and attention (McCoy et al., 2007; Nair et al., 2011). Sleep fragmentation was found to impair spatial learning in rats with a concurrent 32% decrease in the number of neurons in the dentate gyrus of the hippocampus (Sportiche et al., 2010). Further, decreased long term potentiation and increased hippocampal oxidative stress have been found after 3 and 15 days of sleep fragmentation, respectively (Nair et al., 2011; Tartar et al., 2006). Similarly to IH, changes in the responsiveness of wake-active neurons were seen after sleep fragmentation (Y. Li et al., 2014). Neuronal activity was reduced in a range of neuronal populations, including depleted axonal projections from catecholaminergic and orexinergic neurons, and reduced excitability of LC neurons (Y. Li et al., 2014). No studies have specifically examined the effects of sleep fragmentation on A β deposition or tau accumulation.

One study compared the effects on spatial learning and memory in rats of 24 h sleep fragmentation or 24 h IH. They found significant impairment in memory retention and consolidation after sleep fragmentation but no changes after 24 h IH (Ward et al., 2009). They did however find impairments after 3 days of IH. This study demonstrates that sleep fragmentation and IH may differentially affect brain function, with sleep fragmentation having more acute effects. Conversely, a study comparing sleep properties of mice exposed to IH, sleep fragmentation or IH + sleep fragmentation found that the IH and IH + sleep fragmentation groups showed similar changes in their sleep pattern, whereas sleep fragmentation alone elicited weaker changes (Kaushal, Ramesh, & Gozal, 2012). This study suggests that earlier deficits may be attributed to IH rather than sleep fragmentation. Further

research is required to untangle the effects of IH and sleep fragmentation on cognitive function and neurodegenerative changes.

1.3.7 Neuropathological markers in CSF and blood in OSA

Evidence for neuropathological changes in OSA patients has been gathered by investigating markers of neurodegeneration in the blood and CSF. Increased inflammatory markers are regularly seen in OSA patients compared to controls (Alberti et al., 2003; de la Peña Bravo et al., 2007; Nadeem et al., 2013), and while this may suggest the presence of neuroinflammation, other systemic factors also known to increase inflammation, such as obesity and hypertension, are not always accounted for. The levels of the astrocyte marker S100b are increased in the sera of OSA patients (Braga, Martinez, Wofchuk, Portela, & Souza, 2006; Da Silva et al., 2008; Duru et al., 2012), possibly indicating the presence of reactive gliosis in the brain. This marker is also increased in the sera of AD patients (Chaves et al., 2010). The neuronal marker, neuron specific enolase (NSE), which is commonly increased peripherally after brain damage, is not increased in the sera of OSA patients compared to controls (Braga et al., 2006; Da Silva et al., 2008). As S100b levels are increased, it could indicate that reactive gliosis is occurring in order to prevent neuronal damage. Further, the brain injury in OSA could be thought of as being more mild and chronic than the severe and rapid brain injury that characterises a stroke or traumatic brain injury. Rapid onset brain injuries are often accompanied by an increase in NSE in CSF and/ or serum (Ross, Cunningham, Johnston, & Rowlands, 1996; Uzan et al., 1995).

Increased levels of phosphorylated tau and A β have been detected in the sera of OSA patients compared to controls the morning after PSG (Bu et al., 2015). It is notable that increased levels of phosphorylated tau are also detected in the sera and CSF of AD patients (Richens et al., 2014; Schneider et al., 2009). Bu et al. (2015) found a significant positive correlation between the amount of serum A β and the severity of OSA. While this relationship seemingly provides further support for AD-like neuropathological changes in OSA, AD patients actually show reduced serum A β levels, specifically A β 42 (Richens et al., 2014; Schneider et al., 2009). On the other hand, patients with MCI do show an increase in serum A β 42 levels, suggesting that the prodromal phase of AD is characterised by an increase in serum A β 42, followed by a decrease as the disease progresses (Cammarata et al., 2009). One study found decreased CSF A β and reduced slow wave activity during sleep in OSA patients when compared to healthy controls (Ju et al., 2016). This result is similar to the decreased levels of

CSF A β seen in AD, which are thought to be the result of increased deposition of A β in brain tissue.

1.3.8 Neuropathology in OSA (human brain tissue)

Only one study has investigated the neuropathological changes in OSA patients by looking directly at *post-mortem* brain tissue (Gelber et al., 2015). This study investigated the presence of a range of brain lesions including A β plaques, NFTs, gliosis and neuronal loss. However, this study focused on one brainstem nucleus (LC) and cerebral cortex, and did not investigate the hippocampus, which has been highlighted as one of the most vulnerable regions in both OSA and AD. Gelber and colleagues found no association between OSA sleep variables and the presence of A β plaques or NFTs in cerebral cortex and did not investigate these in the LC. However, a relationship was found between time spent with reduced arterial oxygen desaturation and an increase in brain microinfarcts over four cortical regions. A correlation was found between the oxygen desaturation level and the amount of gliosis and neuronal loss in the LC, but no other regions were investigated for this type of cellular injury. While this study provided an important first step in the investigation of AD-like pathological changes in OSA, many questions remain unanswered.

1.4 Conclusion

Individuals with OSA in midlife are much more likely to develop AD in later life, while individuals with AD are very likely to have comorbid OSA. A substantial body of evidence indicates that many parallels exist between the cognitive and neurological changes that occur in OSA and AD, yet until now, there has been only one neuropathological study of *post-mortem* tissue from OSA patients. Further, the cognitive deficits, blood/CSF marker changes and brain alterations seen in AD are similar to those seen in OSA, despite most neuropathological data being obtained from animal models. These features include A β deposition, NFT accumulation, neuronal loss, reactive astrogliosis, inflammation and white matter degeneration. While these factors have been found to be associated with OSA indirectly, it remains to be seen whether these neuropathological changes are occurring in the brains of OSA patients. If they are, then it has significant implications for the implementation of treatment such as CPAP, which may be able to slow if not prevent the neuropathological changes associated with OSA and AD. Given the links between these two disease states, and

the treatability of OSA, there is an evident need to investigate the neuropathological changes in *post-mortem* tissue from OSA patients, especially in the hippocampus and overlying cortical areas, where the neurodegenerative changes in AD are thought to begin.

1.5 Thesis aim

The aim of the present thesis was to investigate the neuropathological changes in *post-mortem* tissue from OSA patients. Specifically, AD neuropathology (A β plaques and NFTs), reactive gliosis, hippocampal atrophy and demyelination were investigated in relation to OSA severity (ODI). Further, the effect of CPAP treatment and patient age were also investigated in relation to the various neuropathological features.

Chapter 2 Materials and methods

All three projects of this thesis used *post-mortem* human brain tissue collected at autopsy and stored in archives in Iceland. Through collaboration with Professors Thorarinn Gislason and Bryndis Benediktsdottir of Landspítali University Hospital, Iceland the de-identified brain tissue samples and corresponding medical records were sent to RMIT, Melbourne, Australia. This project was approved by the National Bioethics Committee, Iceland, reference number 09-087-CM (Appendix A) and the RMIT Human Research Ethics Committee with reference number ASEHAPP 71-16 (Appendix B).

2.1 Materials

Ethanol, sodium chloride, sodium dihydrogen orthophosphate, disodium hydrogen phosphate and sodium acetate were sourced from Merck Millipore. Bovine albumin serum (BSA), donkey serum, goat serum, cresyl violet acetate, glacial acetic acid, formic acid, diaminobenzidine-nickel sulphate (DAB), ethylenediaminetetraacetic acid (EDTA), trizma hydrochloric acid (Tris-HCl), sodium dodecyl sulphate (SDS) were sourced from Sigma Aldrich Australia. Ethanolamine and hydrogen peroxide (H₂O₂) were from BDH. Histolene and Depex were from Gracle HSD. Triton X-100 was from APS Finechem, Reveal It antigen retrieval solution was from ImmunoSolutions and streptavidin-biotinylated horseradish peroxidase (RPN1051) was from GE Healthcare.

2.2 Subjects/participants

Diagnosis of OSA and provision of CPAP treatment started in autumn 1987 in Iceland. The OSA diagnoses are based on whole night polysomnography (PSG) studies involving oximetry and a variable number of other parameters. Determination of OSA severity has been based on the number of apnoeas and hypopneas per hour (AHI), but defined somewhat differently since 1987. The parameters that typically define an apnoea or hypopnea, such as the minimum length of time and the percentage decrease in airflow, have changed considerably over time (Gislason, 2014). However, a 4% decrease in oxygen saturation has always been required to calculate the number of oxygen desaturations per hour (ODI). Due to the archival nature of this study, not all of the AHI records could be retrieved (and were not

fully comparable), whereas all of the ODI records were recovered. Therefore ODI is used in this study as the measure of OSA severity.

In March 2014 a total of 8,853 OSA patients 18 years and older had been registered with the diagnosis of OSA in Iceland (total population 320 000) since 1987; among them 7,531 were alive, 5,456 males and 2,075 females. Among the deceased, 121 underwent autopsy at the Department of Pathology, Landspítali University Hospital and 61 of these autopsies included brain tissue that had been preserved and stored. Permission was granted by the Landspítali University Hospital and the National Bioethics Committee, Iceland, for these brain samples to be sent to RMIT University, Melbourne, Australia for research purposes. Patients were excluded from this study if they had been diagnosed with dementia (including Alzheimer's disease), if their clinical records could not be found, if no hippocampal tissue was available or if evidence of hemorrhagic stroke could be seen in the area of interest. Micrographs from 1 patient with clinically diagnosed Alzheimer's disease are included in Chapter 3 for visual comparison purposes only and no data from this patient was included in the analysis.

The Department of Respiratory Medicine and Sleep at the Landspítali University Hospital has been the sole provider of CPAP treatment in Iceland since 1987. CPAP treatment has been the first line of treatment and upper airway surgery has seldom been performed since 1992 except to correct anatomical deformities. Icelandic CPAP users are all recorded in a constantly-updated register, and whether or not patients regularly used CPAP is included in their medical records. This information was included in the present study to assess the impact of CPAP use.

Ideally, a control group consisting of people without OSA would have been used in this project, however that was not possible. An adequate control subject must have undergone the same diagnostic tests (overnight PSG) as OSA patients and obtained a negative diagnosis (AHI or ODI <5 events per hour sleep). This is important in OSA research, as undiagnosed OSA is highly prevalent (Simpson et al., 2013; Singh et al., 2013), therefore the chances of randomly selecting a control group of participants without OSA is extremely unlikely. Two subjects included in the present study had ODI scores <5 and would qualify as controls, however two is not a large enough sample for a control group. For this reason, the present study contains no control group. Statistical analyses were chosen that enabled an investigation of the relationship between severity of OSA (ODI score) and neuropathological variables.

2.2.1 Study sample

Of the 61 patients with preserved brain tissue that was sent to RMIT University, 35 patients had tissue from the hippocampus, one patient diagnosed with AD was excluded from all analysis, therefore the final sample used for the present study was 34. The first project (Chapter 3) also used tissue from the brainstem at the level of the pons, of the original 61 brains, 25 of these contained tissue from the brainstem. Some patients were excluded from some of the studies due to various reasons, the sample size for each part of each project is outlined in Table 2.1.

The final study sample consisted of 34 patients (18 females and 16 males). OSA severity, as measured by the ODI ranged from 1.9-92.2 desaturations/h sleep. T-tests comparing male and female participants found no significant differences in the ODI, age, BMI or interval from diagnosis to death. Among these 34 patients, 18 were known to have regularly used CPAP until they died. Of the remainder, 3 patients were known not to have ever used CPAP, while 13 patients were not using CPAP at the time of death but may or may not have utilized CPAP for some period of time between diagnosis and death (older records and uncertain). For the purposes of the present study, only those patients known to have regularly used CPAP were included in the 'CPAP-users' group. All other patients were considered to be 'CPAP non-users'. The mean interval between diagnosis and death for the CPAP-users was 7.1 ± 6.2 years, and while these patients were known to have used CPAP machines up until their death, data regarding the extent of nightly usage are not available.

For each of the three projects, the total sample described above was used. Descriptive statistics of the samples are included within the relevant chapters. For project 1 (AD markers; Chapter 3) brainstem tissue was also used. Entorhinal cortex tissue was not available from all patients and therefore some analyses in Chapters 4 (Glia) and Chapter 5 (Hippocampal size and demyelination) have a smaller sample size for the entorhinal cortex, compared to those for the parts of the hippocampus.

Table 2.1 Sample sizes for different projects.

Project/Chapter	Sample size	No. of excluded samples	Reason for sample exclusion
AD neuropathology (Chapter 3)	34	0	
Reactive gliosis – hilus, CA3, CA1 (Chapter 4)	34	0	
Reactive gliosis – entorhinal cortex (Chapter 4)	28	6	Insufficient entorhinal cortex tissue
Hippocampus size – dentate gyrus & CA1 (Chapter 5)	32	2	Multiple sclerosis
Hippocampus size – entorhinal cortex (Chapter 5)	26	2 6	Multiple sclerosis Insufficient entorhinal cortex tissue
Myelin staining – dentate gyrus & CA1 (Chapter 5)	31	2 1	Multiple sclerosis Tissue damage
Myelin staining – entorhinal cortex (Chapter 5)	25	2 1 6	Multiple sclerosis Tissue damage Insufficient entorhinal cortex tissue

2.3 Brain sample collection

Brains were dissected at autopsy. Details of the *post-mortem* interval are unknown. Samples of tissue were post-fixed in 10% formalin. The tissue blocks were subsequently dehydrated in graded ethanols, embedded in paraffin wax and archived. Blocks from the medial part of the temporal lobe (including rostral hippocampus and parahippocampal gyrus) and the brainstem pons (including LC) were selected for use in the present study.

2.4 Sectioning

After being transported to RMIT University, the tissue blocks were sectioned at 20 μ m thickness on a microtome, dried onto glass microscope slides, then dewaxed in Histolene (2 changes) and rehydrated in graded ethanols (100% 2 changes, 95%, 70%, H₂O). Note that paraffin sections of brain are more commonly cut at a thickness of 5-10 μ m, however 20 μ m was selected for the present study as the greater thickness affords superior visualization of cellular detail. Immunohistochemistry was performed using a variety of markers as well as cresyl violet histology (detailed below).

2.5 Histology

2.5.1 Immunohistochemistry

The immunohistochemistry protocol followed that previously described (Robinson, 2001). After deparaffinisation, sections underwent antigen retrieval to reveal antigenic sites (Table 2.2). Sections were then incubated with blocking solution (0.1 M phosphate buffered saline (PBS), 1% BSA, 1% Triton X-100, 1% ethanolamine and 4% serum) for 3 h at room temperature. Serum from the host animal of the secondary antibody was used in the blocking solution, primary and secondary antibody dilutants to reduced non-specific background staining. The blocking step was followed by incubation in primary antibody solutions overnight at room temperature. Primary antibodies were diluted at the concentrations specified in Table 2.2. These concentrations were selected following optimisation experiments conducted previously using tissue from cortical brain regions of OSA patients.

Primary antibody dilutant consisted of 0.1 M PBS, 1% BSA, 0.5% Triton X-100 and 4% serum. Sections were subsequently incubated for 3 h with appropriate secondary antibodies diluted at 1:300 (Table 2.2). Secondary antibody dilutant consisted of 0.1 M PBS, 1% BSA and 4% serum, followed by streptavidin-biotinylated horseradish peroxidase diluted at 1:300 in PBS and 1% BSA. Between each of the above steps, sections were washed for 3 x 10 min in 0.1 M PBS. After incubation with streptavidin-biotinylated horseradish peroxidase, sections were washed for 2 x 5 min in 0.1 M PBS then for 2 x 10 min in 0.175 M sodium acetate buffer (NaOAc). Sections were then incubated with the chromagen DAB diluted in NaOAc buffer at 0.05% for 10 min, then 0.05% DAB in NaOAc buffer with 0.004% H₂O₂ for 15 min. Immunolabelled sections were then washed in NaOAc buffer for 2 x 5 min then 0.1 M PBS for 2 x 10 min and left overnight in 0.1 M PBS at 4°C. On the following day, sections were dehydrated in graded ethanols (70%, 95%, 100%; 2 changes in 100% only) and Histolene (2 changes) before being coverslipped with Depex. After being left to dry, sections were viewed under the microscope.

Table 2.2 Antibody dilution and antigen retrieval techniques used for immunohistochemistry.

Antibody (company)	Product no.	Dilution	Antigen retrieval	Secondary antibody	Product no.	Secondary incubation time
Anti Tau (Sigma)	T6402	1:500	None	Donkey anti rabbit (GE Healthcare)	RPN1004	3 h
Anti β amyloid (DAKO)	M0872	1:100	1 h in 99% formic acid	Donkey anti mouse (Merck Millipore)	AP192B	3 h
Anti GFAP (DAKO)	Z0334	1:20000	30 min Reveal It solution approx 80°C	Donkey anti rabbit (GE Healthcare)	RPN1004	4 h
Anti glutathione synthetase (Sigma)	G2781	1:2500	None	Donkey anti rabbit (GE Healthcare)	RPN1004	4 h
Anti s100β (Abcam)	ab52642	1:5000	30 min Reveal it solution approx 80°C	Donkey anti rabbit (GE Healthcare)	RPN1004	4 h
Anti CD11β (Merck Millipore)	MABT149	1:1000	30 min Reveal it solution approx 80°C	Donkey anti rabbit (GE Healthcare)	RPN1004	4 h
Myelin basic protein (Abcam)	Ab7349	1:1000	40 min EDTA buffer (Syrbu & Cohen, 2011) approx 80°C	Goat anti rat (Merck Millipore)	AP183B	6 h

2.5.2 Cresyl violet

After deparaffinisation, sections were incubated for 20 min at room temperature in 0.5% cresyl violet solution. Sections were washed briefly in H₂O, then in 70% ethanol and then differentiated in 95% ethanol + glacial acetic acid for 5 min. Slides were then dehydrated in 100% ethanol (2 changes) and Histolene (2 changes) before being coverslipped with Depex. After being left to dry, sections were viewed under the microscope.

2.6 Microscopy

All sections were viewed on an Olympus AX-60 microscope, all micrographs used for analysis were taken with a Olympus DP73 camera attached to the AX-60 at the magnification specified in the relevant chapter. Image analysis was performed with the Olympus software CellSens. Some low magnification images were taken using an Olympus slide scanner VS120-S5; these images were used for illustrative purposes, not for analysis.

2.7 Histology analysis

Each of the following chapters examines different histological features of the brain. As this necessitated the use of different methods of image analysis, these details are included in the relevant chapter rather than in the present one.

2.8 Statistical comparisons

Correlational analyses were used for most of the data presented. The limited sample size meant that regression analyses controlling for factors such as age and BMI were not feasible due to low statistical power. A significant correlation between age and ODI was found for the sample (see descriptive statistics in Chapters 3, 4 and 5). Subsequently, correlations were performed between age and any neuropathological variable that was found to be significantly related to ODI. This allowed for a general indication of whether age was likely to contribute to the relationship. If age was not correlated with the neuropathological variable, then it is unlikely that age influenced the relationship with ODI. Conversely, if age was correlated with the neuropathological variable, then it is likely that age influenced the relationship with ODI. Without performing multifactorial analyses the precise contribution of age cannot be determined, however this method provided an indication of such relationships. All the variables were considered to be independent from each other therefore corrections for multiple comparisons were not required and α was set at $p < 0.05$.

Normality tests showed that ODI had a non-normal distribution and one outlier (ODI = 92.2), in order to normalise this variable a log transformation was performed. This resulted in a normal distribution with no outliers, therefore log ODI was used for all analyses.

Note: The following three data chapters have been written and structured as draft manuscripts to be submitted for publication shortly after the examination of this thesis. For this reason, there may be areas of repetition between the three chapters.

Chapter 3 Alzheimer's disease neuropathology in the hippocampus and brainstem of obstructive sleep apnoea patients

Abstract

Obstructive sleep apnoea involves intermittent oxygen desaturations during sleep. Patients experience significant impairments including memory deficits and reduced hippocampal volume. Memory deficits and reduced hippocampal volume are also seen in AD patients, accompanied by cumulative pathogenesis of NFTs and A β plaques in the hippocampus and brainstem. It is not known if such pathogenesis occurs in OSA patients, despite OSA being a risk factor for AD. The present study used immunohistochemistry to investigate post-mortem tissue of OSA patients for the presence of NFTs and A β plaques. It was found that increasing OSA severity was significantly related to the burdens of NFTs ($r^2=0.166$, $p=0.017$) and A β plaques ($r^2=0.134$, $p=0.033$) in the hippocampus, but not in the brainstem. Further, CPAP was found to be protective against pathogenesis in the hippocampus. Patients that used CPAP had reduced burdens of both NFTs and A β plaques, whereas CPAP non-users had greater burdens. These findings have important implications for the treatment of OSA and possibly AD. This study has shown that increasing severity of OSA is associated with increasing burdens of NFTs and A β plaques in the hippocampus and overlying cortex, consistent with the pathogenesis of AD.

3.1 Introduction

OSA involves intermittent cessations of breathing during sleep, leading to transient arterial oxygen desaturations and arousals. Estimates of prevalence range from 3-10% in adults younger than 40 years of age to 10-20% in older adults (Gislason, 2014; Punjabi, 2008; Simpson et al., 2013). A recent review suggested the prevalence of moderate-severe OSA may be as high as 36% in adults over 60 years of age (Senaratna et al., 2017). CPAP during sleep provides effective symptomatic treatment (Buchanan & Grunstein, 2011). OSA patients frequently display deficits in attention, memory and executive functioning (Torelli et al., 2011). While these deficits improve with CPAP treatment, the extent of recovery is less complete in patients with moderate-severe OSA (Canessa et al., 2011), suggesting that severe OSA may permanently injure the brain. The hippocampus is particularly sensitive to hypoxic injury (Di Paola et al., 2008), and MRI studies of OSA patients have revealed degenerative

changes in the hippocampus, as well as in nearby structures such as the amygdala, parahippocampal gyrus and ventral parts of the temporal cortex (Canessa et al., 2011; Macey et al., 2008), regions commonly affected in AD.

A possible relationship between OSA and AD is indicated by clinical studies that have shown OSA to be overrepresented in patients with dementia. For instance, a retrospective study of 485 dementia patients (aged 65-99) who had been hospitalized for falls, found that 42% had moderate-severe OSA, as defined by an ODI of >15 events per hour (Frohnhofer & Roffe, 2012). This is more than double the estimated prevalence of OSA in people aged over 65 years (Young, Peppard, & Gottlieb, 2002). Other studies have reported that more than 50% of institutionalized dementia patients have sleep-disordered breathing, which is predominantly OSA (Guarnieri et al., 2012). Furthermore, prospective population-based studies have reported that OSA is an independent risk factor for the development of dementia in cognitively normal elderly persons (Chang et al., 2013; Yaffe et al., 2011).

AD is characterised by executive dysfunction, a progressive deterioration of memory, particularly short-term memory, and reduced volume of the hippocampus and parahippocampal gyrus (Rombouts et al., 2000). The hippocampal atrophy is accompanied by an accumulation of A β plaques and NFTs, which first accumulate in the rostral part of the parahippocampal gyrus and then spread to the adjacent hippocampus and other cortical regions (Braak et al., 2006; Braak & Braak, 1991; Burns & Iliffe, 2009; Thal et al., 2000). The causative agent(s) of AD is(are) so far unknown, however the pattern of hippocampal pathology is well characterised, although there remains some debate as to which neuropathological feature occurs first, A β or NFTs (Braak & Del Tredici, 2015; Selkoe & Hardy, 2016).

Like in AD, patients with severe OSA exhibit hippocampal atrophy, but it is not known whether the atrophy is accompanied by the presence of A β plaques and NFTs. It is notable that studies using chronic IH to model OSA in rats, as well as studies of AD-transgenic mice have demonstrated that IH leads to a significant increase in the cerebral and hippocampal burdens of A β (Ng, Lau, & Fung, 2010; Shiota et al., 2013). Further, the initial stage of NFT development, tau phosphorylation, is promoted by hypoxic conditions (Fang et al., 2010; C. E. Zhang et al., 2014). Together these animal studies show that IH is capable of precipitating neuropathological changes that resemble those seen in AD, however it remains to be seen

whether the presence of OSA in humans increases the incidence of A β plaques and NFTs in the hippocampus.

While age is the most significant risk factor for the development of AD, a genetic predisposition exists. It is well established that the presence of Apo E4 alleles are a genetic risk factor for AD. It was thought that Apo E4 may be a genetic risk factor for OSA, with one study showing that a significantly higher percentage of Apo E4 carriers had moderate-severe OSA (AHI ≥ 15) when compared to non-Apo E4 carriers (Kadotani et al., 2001). However a more recent meta-analysis concluded that there is no increased risk (Varvarigou, Dahabreh, Malhotra, & Kales, 2011). Despite this, it is important to know the Apo E genotype of people with OSA in order to take into account any effect it may have on the development of A β plaques and NFTs.

In AD, neurodegenerative changes have been observed in the brainstem, particularly in the LC and dorsal raphe nucleus (Giess & Schlote, 1995). Studies have shown hyperphosphorylated tau, NFTs and A β plaques in the brainstem of AD patients (Parvizi et al., 2001; Rüb et al., 2000) and there are suggestions that these changes may occur early in the pathogenesis of the disease, possibly prior to the appearance of these features in the hippocampus (Braak & Del Tredici, 2015). However, these neurodegenerative changes have not been studied in as much detail as the hippocampal pathology, and the specific spatiotemporal pattern of accumulation in the brainstem is yet to be fully elucidated. Interestingly, the LC and dorsal raphe nucleus both play a role in maintaining wakefulness; the neurons in both nuclei are active during wakefulness and inactive during both slow wave sleep and REM sleep (Murillo-Rodríguez et al., 2012). Wake-activated neurons of the LC have been shown to be impaired or lost entirely in animals exposed to chronic IH or sleep fragmentation, which are both models of OSA (Y. Li et al., 2014; Zhu et al., 2007). Neuronal degeneration has also been shown in the LC and dorsal raphe nucleus of guinea pigs exposed to IH (J. H. Zhang et al., 2010). Further, the only study to investigate human neuropathology in OSA found that the extent of neuronal loss in the LC was associated with the extent of decrease in blood oxygenation levels during sleep (Gelber et al., 2015). However, the presence of A β plaques and NFTs in the LC of OSA patients has not yet been investigated.

It is possible that OSA facilitates the onset of AD. A previous study examined autopsied brain tissue from four neocortical regions of 167 men who had died within ten years of an overnight PSG test, for the presence of A β plaques and NFTs (Gelber et al., 2015), and they

concluded that OSA is unlikely to contribute to AD pathogenesis. However, their study did not examine the hippocampus and parahippocampal gyrus, where the first neuropathological changes of AD are typically identified. Further, their investigation of the brainstem was restricted to one nucleus (LC) and it did not investigate whether A β and NFTs are present in other brainstem nuclei.

The present study examined autopsied brain tissue from patients with clinically-verified OSA, to determine whether OSA severity is associated with an increased burden of A β and NFTs. The regions examined were the rostral pons at the level of the LC, and the hippocampus, including the parahippocampal gyrus and adjacent cerebral cortex. Age, Apo E status and CPAP use were investigated for their potential to contribute to any observed effects.

3.2 Methods

3.2.1 Study sample

The study sample consisted of 34 of the 61 patients from the Iceland cohort described in Chapter 2. These 34 patients all had autopsy tissue from the medial part of the temporal lobe, including the hippocampus and parahippocampal gyrus. A second sample of 25 patients had autopsy tissue from the brainstem at the level of the rostral pons, including the LC.

To investigate the frequency of different types of neuropathology as a function of OSA severity, the sample was split at the median ODI value of 18.6. Those with an ODI less than 18.6 were considered for the purpose of this study to have mild-moderate OSA (low OSA) and those with an ODI greater than 18.6 were considered to have moderate-high OSA (high OSA).

3.2.2 Tissue processing

Brain tissue used in this study was processed as described in Chapter 2.

3.2.3 Immunohistochemistry

The immunohistochemistry protocol followed that previously described in Chapter 2. Briefly, after deparaffinisation, sections to be stained for A β underwent antigen retrieval in which sections were incubated in 99% formic acid for 1h. Sections to be stained for tau did not

require antigen retrieval. After blocking, sections were incubated with primary antibodies for anti- β -amyloid (DAKO, M0873) diluted at 1:100 or anti-tau (Sigma, T6402) diluted at 1:500. Following this, sections were incubated in appropriate secondary antibodies for 3h then processed as previously described (Chapter 2).

All brain sections were processed in an identical manner. The examiner (JO) was blinded to the OSA severity of the patients when staging the brain tissue for burden of A β plaques and NFTs. The second examiner (SR) was also blinded to the OSA severity and confirmed the classification of the A β plaques and NFTs. For each brain, two hippocampus sections were analysed for each antibody. The classifications were based on the highest stage or phase reached in the two sections. For brainstem sections, one section was analysed for each brain, due to the limited availability of tissue.

3.2.4 Classification of hippocampal NFT burden

Phosphorylated tau was identified by immunopositive staining for the anti-tau antibody, evident as NFTs, neuropil threads and neuritic plaques. The location and density of NFTs was used for classification in the hippocampus in accordance with previous findings (Braak et al., 2006). Sections were examined using bright field microscopy to determine NFT burden according to the stages defined by Braak et al (2006) for AD pathogenesis. If no NFTs could be seen in the medial part of the temporal lobe, the section was classified as Stage 0. If NFTs were limited to the transentorhinal region (between the parahippocampal gyrus and the occipitotemporal gyrus), then the section was classified as Stage 1. If NFTs could also be seen in the entorhinal cortex and CA1/CA2 of the hippocampus, then they were classified as Stage 2. When NFTs were present in CA3/CA4 and the occipitotemporal gyrus, they were classified as Stage 3. Where a substantial density of NFTs was present in the areas affected in Stage 3, they were classified as Stage 4. Figure 3.1 illustrates the staging classification.

Regional changes in NFT burden were assessed by counting the number of NFTs in 5 regions of the hippocampus and surrounding cortex: transentorhinal region, entorhinal cortex, CA1, hilus and occipitotemporal cortex (OTC). These regions were chosen because they corresponded to the staging classifications detailed above.

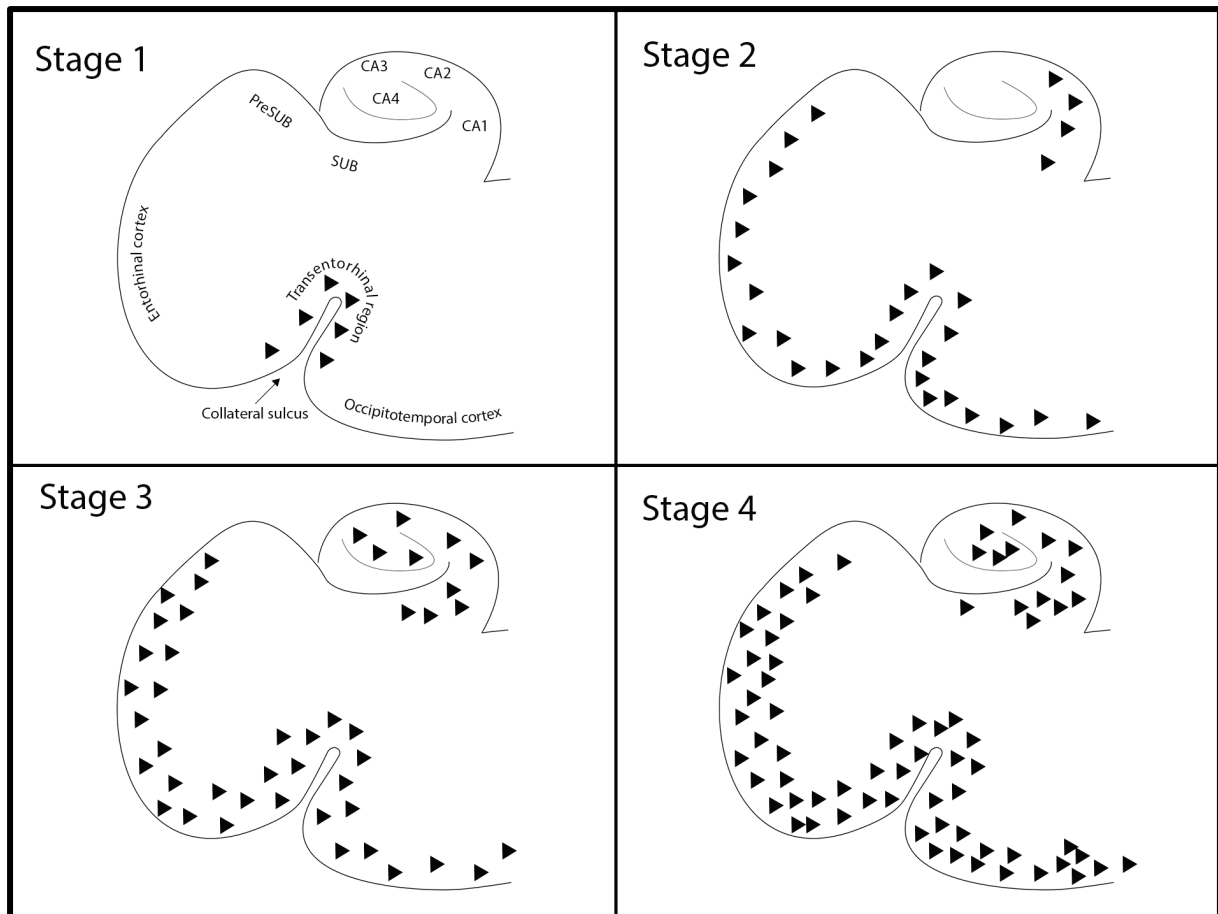


Figure 3.1 Illustration of NFT classifications. Stage 1 has NFTs only in the transentorhinal region; Stage 2 has NFTs in the entorhinal cortex and CA1/CA2. Stage 3 has NFTs in CA3/CA4, while Stage 4 has a substantially higher density of NFTs in all affected regions. Black triangles indicate the presence of NFTs in that region. The illustration is based on the description of NFT stages from Braak et al. (2006).

3.2.5 Classification of hippocampal $A\beta$ plaque burden

$A\beta$ plaques were staged according to the criteria described by Thal and colleagues for AD pathogenesis (Thal et al., 2000). If no plaques could be seen, then the section was given a classification of Phase 0. Brains were classified as Phase 1 if $A\beta$ plaques were limited to the parahippocampal gyrus and/or the occipitotemporal gyrus. When plaques were present in the subiculum and CA1 as well as in the parahippocampal and/or occipitotemporal gyrus, the brain was classified as Phase 2. If plaques extended into CA2/CA3 and the molecular layer of the dentate gyrus, then the brain was classified as Phase 3. If plaques were also present in the

hilus and throughout CA4, then the brain was classified as Phase 4. Figure 3.2 illustrates the staging classification.

Regional changes in A β plaque burden were assessed by counting the number of A β plaques in 5 regions of the hippocampus and surrounding cortex: transentorhinal region, entorhinal cortex, subiculum, CA1 and hilus. These regions were chosen based on the phase classifications detailed above and differ from the NFT regions examined because there are important regional distinctions between the spatiotemporal patterns of NFTs and A β plaques.

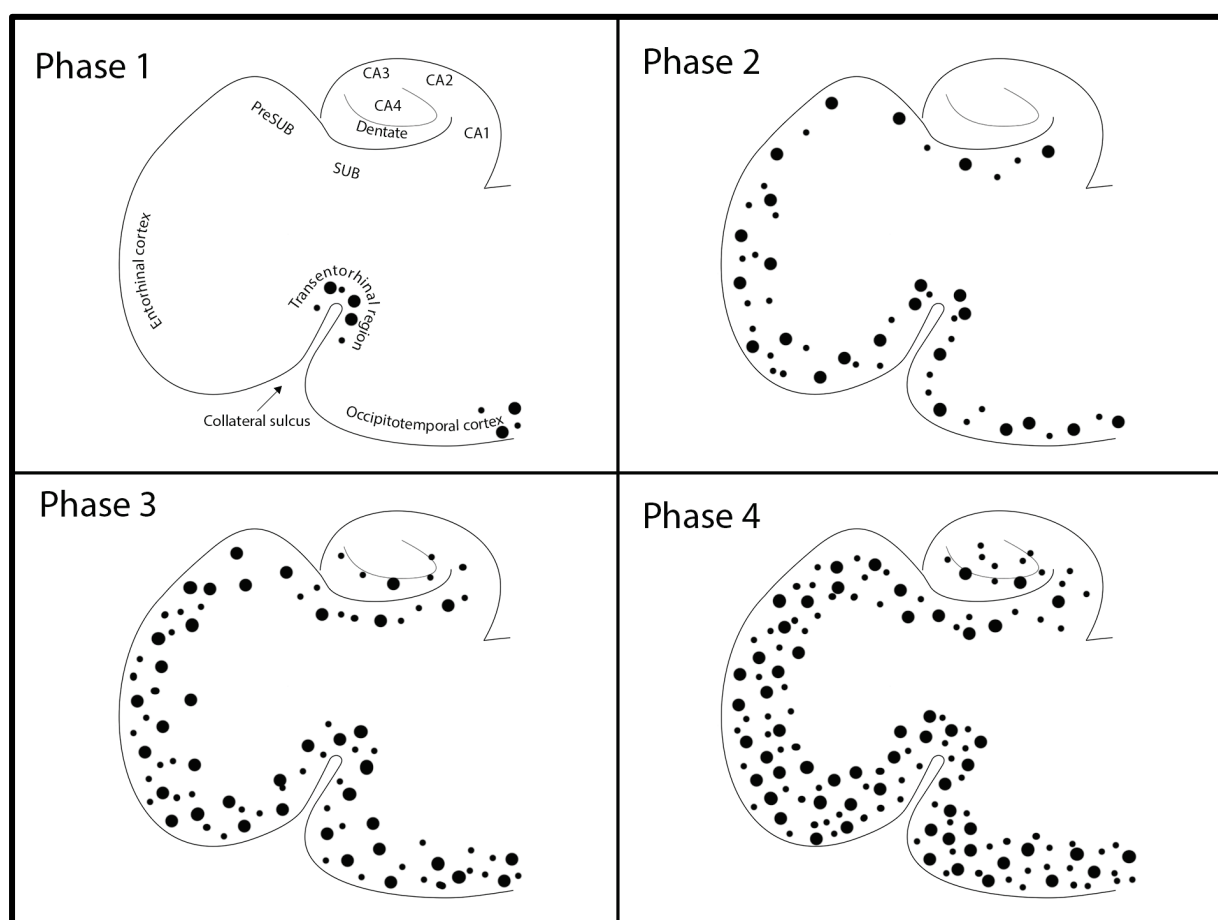


Figure 3.2 Illustration of A β plaque classifications. Phase 1 has A β plaques in the transentorhinal region and/or occipitotemporal cortex; Phase 2 has A β plaques throughout the entorhinal cortex, subiculum and CA1. Phase 3 has A β plaques in the molecular layer of the dentate while Phase 4 has A β plaques throughout CA4. Black circles indicate the presence of A β plaques in that region, with larger circles indicating larger plaques. The illustration is based on the description of A β plaque burden from Thal et al. (2000).

3.2.6 Classification of pathology in brainstem

There is no established classification system for NFT or A β burden in the brainstem. Consequently, the present study conducted simple counts of A β plaques and NFTs in the brainstem sections. The present study identified the locations of NFTs and any other deposits of phosphorylated tau that could be identified. ‘Any phosphorylated tau’ included NFTs, neuropil threads and/or neuritic plaques that were immunoreactive for the anti-tau antibody.

3.2.7 Apo E genotyping

Apo E genotyping was performed at Landspítali University Hospital, Department of Clinical Biochemistry by Elizabeth Cook. I assisted Elizabeth with this procedure during a laboratory visit to Iceland. Apo E genotype was determined based on the Sanger sequencing method (Sanger, Nicklen, & Coulson, 1977). Brain tissue was sectioned as above but sections were not dried onto microscope slides; instead they were collected into Eppendorf tubes. The samples were de-waxed, followed by DNA isolation and amplification using the polymerase chain reaction (PCR). The two oligonucleotides used for DNA amplification, both from TAG (Copenhagen), were:

Apo E sequence F	5' GCGGACATGGAGGACGTG-3'
Apo E sequence R	5' CCCCggcCTGGTACACTG-3'

After successful DNA isolation and amplification samples were sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) to determine the amino acid residues at 112 and 158 which differ by Apo E genotype. Genotype E2/E2 has cysteine residues at both the 112 and 158 position, E2/E3 has cysteine at 112 and cysteine and arginine the 158 position, E2/E4 has cysteine and arginine at both the 112 and 158 positions, E3/E3 has cysteine at 112 and arginine at 158 position, E3/E4 has arginine and cysteine at 112 and arginine at 158 and E4/E4 has arginine at both the 112 and 158 positions (Kontula, Aalto-Setälä, Kuusi, Hämäläinen, & Syvänen, 1990).

3.2.8 Statistical methods

All statistical analysis was performed using the IBM SPSS, except for the Fisher's Exact Test for independence. Fisher's Exact Test was necessary because the requirements for a Chi-squared test were not met. SPSS is unable to perform the Fisher's Exact Test which was

performed using an online calculator that allowed 2x4 and 3x3 contingency table calculations (<http://vassarstats.net>).

3.3 Results

3.3.1 Descriptive statistics

Descriptive statistics for the brainstem and hippocampus samples can be seen in Table 3.1. Correlations were performed between age, BMI, time from diagnosis to death and ODI within each sample. In the brainstem sample no significant correlations were found between any of the descriptive variables. In the hippocampus sample there was a significant correlation between age and ODI ($r=0.416$, $p=0.014$). No other correlations were found between variables. When the hippocampus sample was split into CPAP users and CPAP non-users there was no significant difference between age, time from diagnosis to death or ODI. BMI was significantly higher for CPAP users compared to CPAP non-users ($t(27)=3.5$, $p=0.002$).

Table 3.1 Descriptive statistics of brainstem and hippocampus samples with the hippocampus sample being separated by CPAP use. Mean \pm standard deviation.

	Brainstem sample	Hippocampus sample	CPAP users	CPAP non-users	Significance CPAP users vs. non-users
n	25	34	18	16	
Gender	F=14, M=11	F=18, M=16	F=12, M=6	F=6, M=10	
Age at death (years)	68.8 \pm 11.1	67.0 \pm 11.1	69.9 \pm 11.1	63.8 \pm 10.5	p=0.111
BMI kg/m²	30.7 \pm 6.2 n=21	29.5 \pm 6.2 n=29	32.6 \pm 5.8 n=16	25.7 \pm 4.4 n=13	p=0.002*
Time from OSA diagnosis to death (years)	6.5 \pm 5.3	7.3 \pm 5.8	7.1 \pm 6.2	7.5 \pm 5.7	p=0.815
ODI (events/h)	26.2 \pm 22.2	25.5 \pm 21.3	32.0 \pm 18.8	18.2 \pm 22.0	p=0.057

*p<0.05 CPAP users compared to CPAP non-users

3.3.2 Apo E genotype

Apo E genotype was determined for 26 patients, for the remaining 8 patients, tissue processing at autopsy caused irreversible DNA damage which prevented isolation of DNA and therefore genotyping. Of the genotypes that were determined, most (22/26) were E3/E3, 3 were E3/E4 and 1 was E4/E4. Due to the amount of missing data, this variable was not statistically analysed.

3.3.3 AD pathology in brainstem

Of the 25 brains with sections of pons that included the LC, 6 contained A β plaques and 22 contained phosphorylated tau. The frequency of no pathology, tau only pathology, A β only pathology and tau + A β pathology can be seen in Figure 3.3, separated into those with low and high ODI scores. The low and high OSA groups had similar numbers of patients with

each type of pathology. The percentage of brains with pathology can be seen in Table 3.2 for the low OSA group and the high OSA group. The percentage of brains containing any tau is similar for both low and high OSA. The percentage of brains containing any A β only represents 6 samples. Despite the percentage being higher for those with high OSA, the low number of samples with A β meant that no statistical analysis could be conducted on this variable alone. Instead the tau-only and amyloid-only data were combined into one group (tau or A β) and were used along with the frequencies for no pathology and tau + A β pathology to assess differences in distribution of pathology for low and high OSA. A Fisher's Exact Test for independence found that there was no significant difference between the distribution of pathology for low and high OSA (n=25, p=0.593).

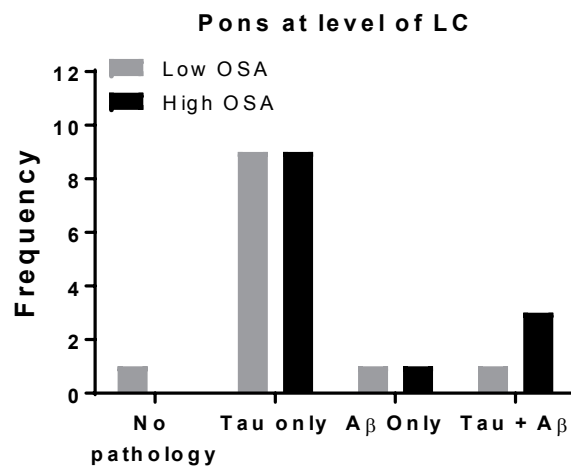


Figure 3.3 Frequency of pathology in the rostral pons at the level of LC, in the low OSA group (ODI<18.6; grey bars) and in the high OSA group (ODI>18.6; black bars).

Table 3.2 Percentage of patients in the low and high OSA groups with tau and/or A β pathology in the pons at the level of the LC.

Pathology	Total sample (n = 25)	Low OSA (n = 12)	High OSA (n = 13)
No pathology	4%	8%	0%
Tau-p only	72%	75%	69%
Aβ plaques only	8%	8%	8%
Tau-p & Aβ plaques	16%	8%	23%
Any Tau-p	88%	83%	92%
Any Aβ plaques	24%	17%	31%

Abbreviations: Tau-p, phosphorylated tau

Due to the low frequency of brains containing A β plaques (8%), no further analysis was conducted. The location of NFTs was further investigated (Figure 3.4). NFTs were seen most frequently in the LC and dorsal raphe nucleus with some present in mesencephalic nucleus of the trigeminal, reticular formation and pontine nuclei (Table 3.3). Figure 3.5 shows high magnification images of tau at the 5 identified locations, NFTs were seen in all locations, whereas neuropil threads were seen mostly in the LC and dorsal raphe nucleus.

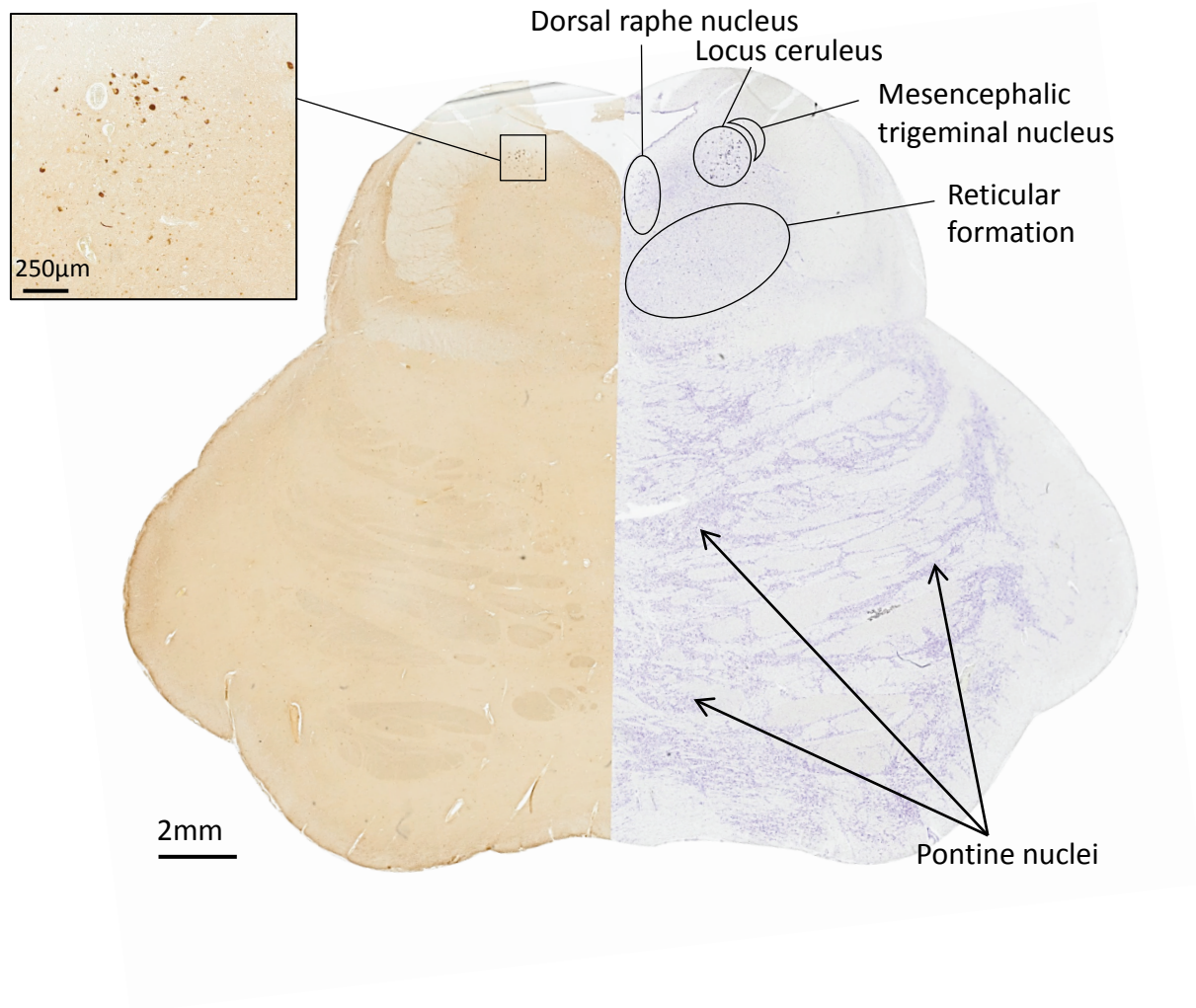


Figure 3.4 Pons at the level of the LC. Hemi-sections stained with cresyl violet (right) and tau (left) including the LC (inset).

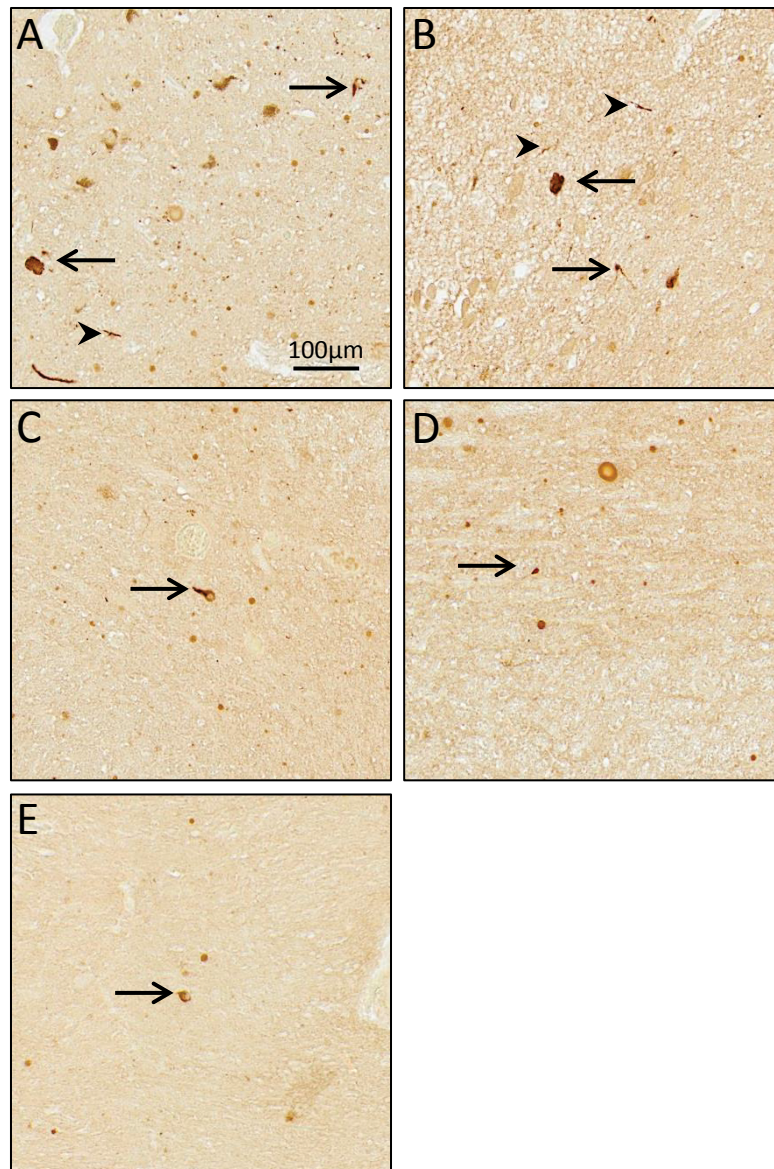


Figure 3.5 Micrographs of NFTs (arrow) and neuropil threads (arrow head) in the LC (A), dorsal raphe nucleus (B), mesencephalic trigeminal nucleus (C), reticular formation (D) and pontine nuclei (E). Scale bar in A applies to all panels.

Table 3.3 Frequency of NFTs and phosphorylated tau in different nuclei of the pons at the level of the LC.

Nucleus	No. of brains with any Tau-p	No. of brains with NFTs
LC	21	17
Dorsal raphe nucleus	17	16
Mesencephalic nucleus of the Trigeminal	5	4
Pontine reticular formation	12	8
Pontine nuclei	6	4

Abbreviations: Tau-p, phosphorylated tau

3.3.4 AD pathology in hippocampus

Hippocampal sections were analysed from 34 brains. Of these, 26 had A β plaques and/or NFTs. 13 brains had NFTs only, while 2 brains had A β plaques only. Both A β plaques and NFTs were observed in 11 of the 34 brains (Figure 3.6A). The distributions of A β plaques and NFTs were analysed, and the brains were then staged for tau and A β , according to the criteria used for staging these neuropathological features in AD. Brains from patients with low OSA more frequently had earlier stages of tau pathology and no amyloid pathology, whereas those with high OSA more frequently had later stages of tau pathology and amyloid pathology (Figure 3.6B & C). The percentage of brains with pathology can be seen in Table 3.4 for those in the low and high OSA groups. In the low OSA group, a higher percentage of brains had no pathology compared to the high OSA group. The high OSA group had a higher percentage of brains with both NFT and A β pathology, as well as more with any NFTs and any A β pathology. Similarly to the brainstem analysis, the tau only and amyloid only frequencies were collapsed into one (tau or A β) and were used along with the frequencies for no pathology and tau + A β pathology to assess differences in distribution of pathology for low and high OSA. A Fisher's Exact Test for independence found a significant difference between the distribution of pathology for low and high OSA (n=34, p=0.004).

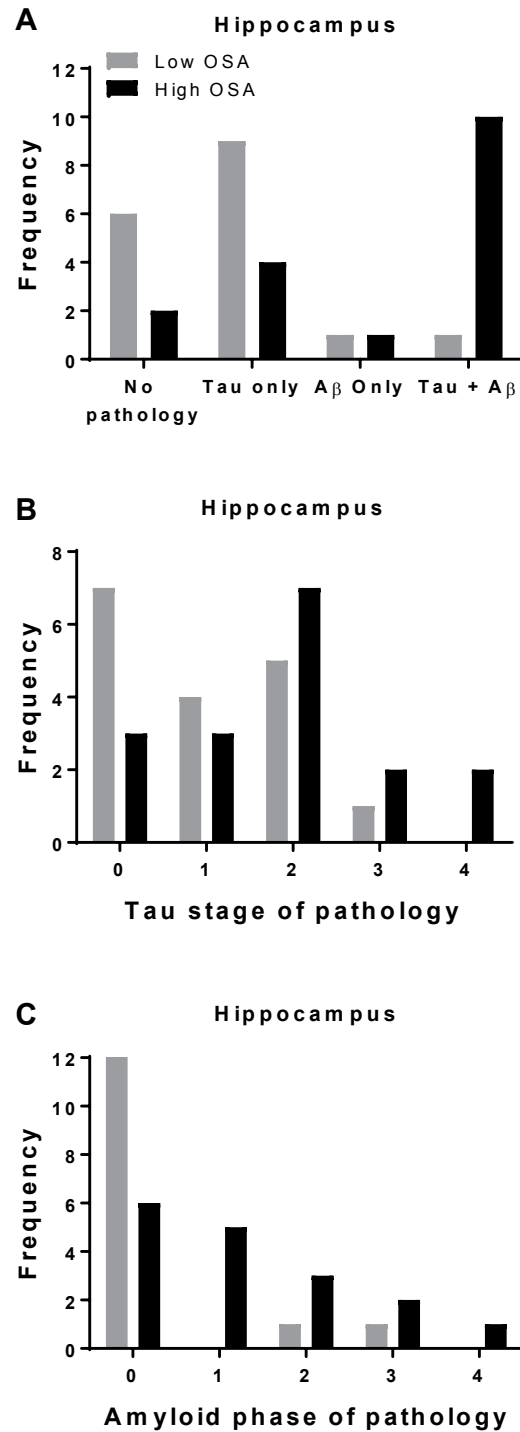


Figure 3.6 Frequency of hippocampus pathology (A) seen in the low OSA group (ODI<18.6; grey bars) and the high OSA group (ODI>18.6; black bars). The frequency of tau stage of pathology (B) and amyloid phase of pathology (C).

Table 3.4 Percentage of patients in the low and high OSA groups with tau and/or amyloid pathology in hippocampus

Pathology	Total sample (n = 34)	Low OSA (n = 17)	High OSA (n = 17)
No pathology	24%	35%	12%
NFTs only	38%	53%	24%
A β plaques only	6%	6%	6%
NFTs & A β plaques	32%	6%	59%
Any NFTs	71%	59%	82%
Any A β plaques	38%	12%	65%

3.3.4.1 NFT burden in the hippocampus

NFTs were observed in 24 of the 34 brains. Of these 24, 7 were at Stage 1, 12 were at Stage 2, 3 were at Stage 3 and 2 were at Stage 4 (Figure 3.6B). Severity of OSA was significantly and positively related to severity of tau burden, this relationship occurred in a log linear manner ($r^2=0.166$, $p=0.017$; Figure 3.7A). This relationship can be seen from the micrographs in Figure 3.8 which show no tau immunoreactivity in a brain with no OSA (Figure 3.8A), a few NFTs in a brain with low OSA (Figure 3.8C), and more NFTs as well as neuropil threads in the brain of a high OSA patient (Figure 3.8E). Figure 3.8 includes images from a patient with AD for comparison. Some of the severe OSA patients resembled AD in the extent of their NFT burden (Figures 3.8E & G).

In order to investigate the effect of CPAP treatment, the group was divided into regular CPAP-users and CPAP non-users and NFT burden was analysed in relation to OSA severity (Figure 3.7C). There was no relationship between OSA severity and NFT burden in CPAP users ($r^2=0.023$, $p=0.548$), whereas there was a significant positive relationship between OSA severity and NFT burden in CPAP non-users ($r^2=0.315$, $p=0.024$). In terms of correlation coefficient (r^2), the relationship between NFT burden and OSA severity for CPAP non-users was stronger than the relationship between OSA severity and NFT burden for the whole sample ($r^2=0.315$ vs. $r^2=0.166$). Further, the relationship for CPAP users was weaker than the relationship for the whole sample ($r^2=0.023$ vs. $r^2=0.166$).

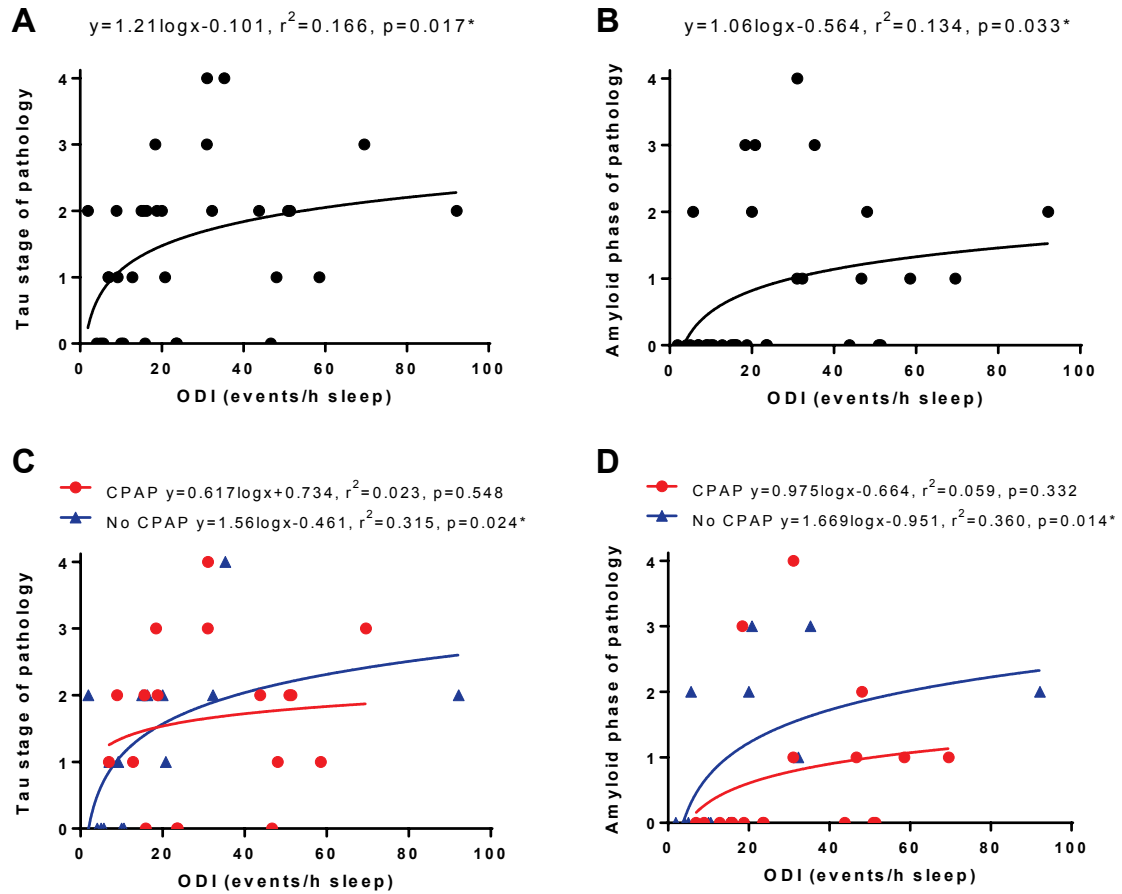


Figure 3.7 Relationship between NFT and A β burden with ODI. Regression of NFT stage (A & C) and A β phase (B & D) with ODI in the total hippocampus sample (A & B) and stratified by CPAP use (C & D).

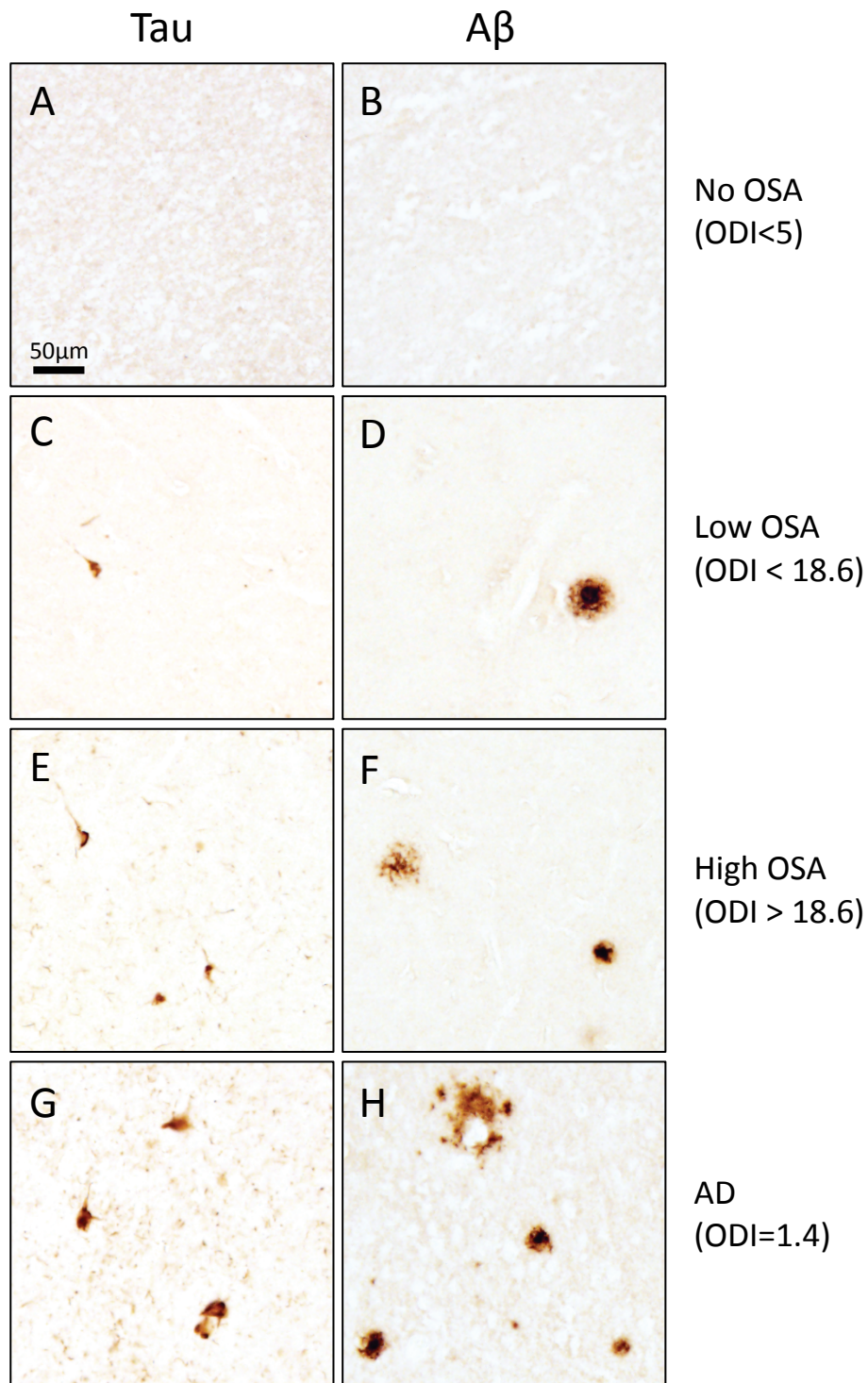


Figure 3.8 Micrographs of hippocampus sections from OSA patients. Patient with no OSA (A, B), low OSA (C, D), high OSA (E, F) or Alzheimer's disease (AD) (G, H). Tau staining indicates NFTs (A, C, E, G) and A β staining indicates amyloid plaques (B, D, F, H). Scale bar in A applies to all panels.

The number of NFTs in 5 different regions of the hippocampus was counted to determine if they differed with OSA severity (Figure 3.9). As the Stage of NFT pathology increased, there were increasing cases with no NFTs in some regions. Cases with no NFTs in a region were excluded from analysis to avoid skewing the data towards positive correlations. For this reason the sample size (n) is different for each region. Positive relationships were seen in the transentorhinal region, CA1 and occipitotemporal cortex (Figure 3.9A, C & E respectively), and no relationships were seen in the entorhinal cortex or hilus (Figure 3.9B & D). The only significant relationship was found in the CA1 region ($r^2=0.231$, $n=20$, $p=0.032$).

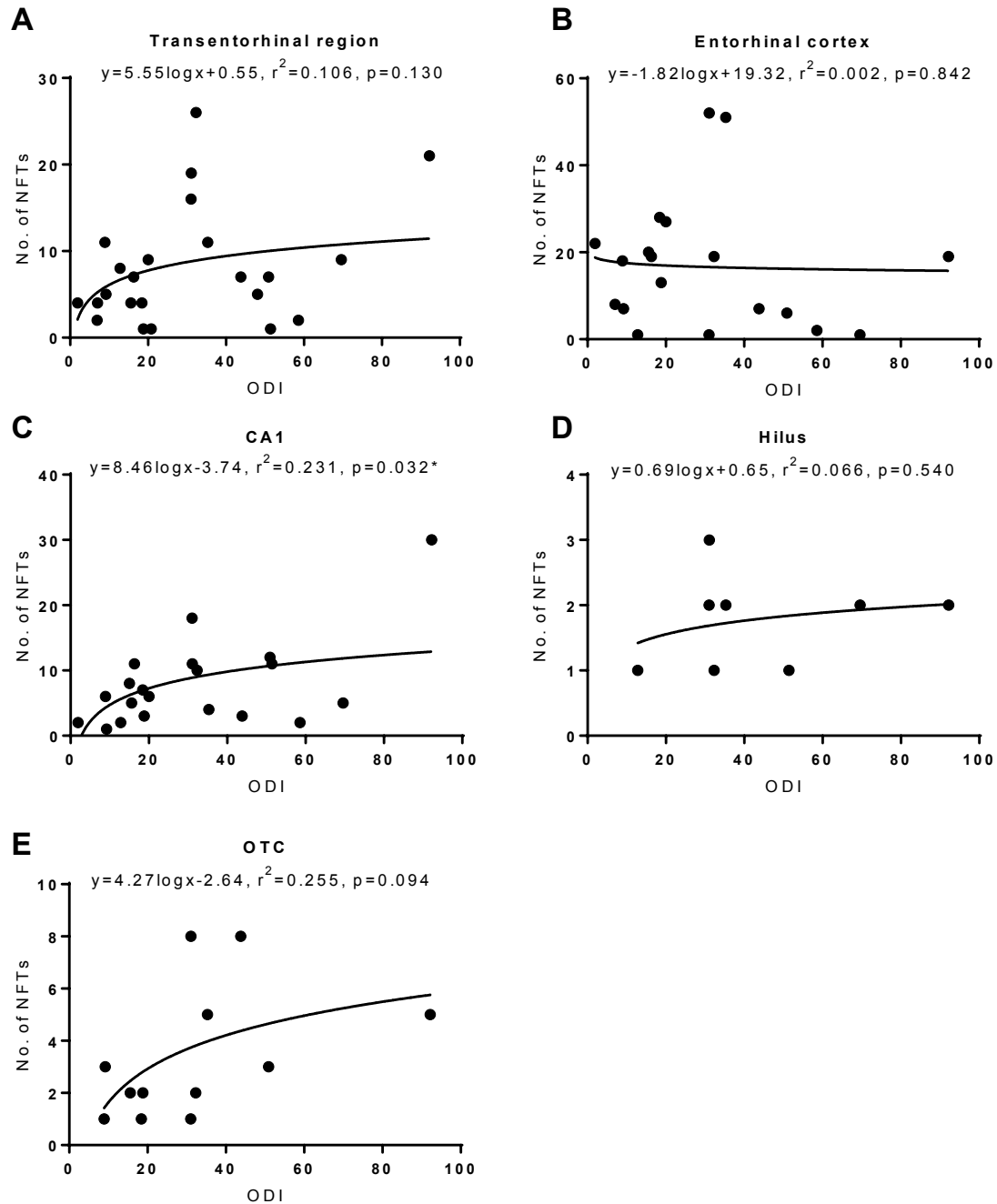


Figure 3.9 Regression between OSA severity (ODI) and NFT count in 5 regions of the hippocampus and surrounding cortex. Transentorhinal region (A), entorhinal cortex (B), CA1 (C), hilus (D) and occipitotemporal cortex (E).

3.3.4.2 $A\beta$ burden in the hippocampus

$A\beta$ plaques were observed in 13 of the 34 brains. Of these, 5 were classified as Phase 1, 4 as Phase 2, 3 as Phase 3, and 1 as Phase 4 (Figure 3.6C). Increasing ODI and severity of $A\beta$

burden were significantly and positively related, and this relationship occurred in a log linear manner ($r^2=0.134$, $p=0.033$; Figure 3.7B). Most patients with an ODI below 20 had no A β burden, whereas an ODI above 20 was associated with a steady increase in pathology burden as ODI increased. Figure 3.8 shows no pathology in brain tissue from a patient with no OSA (Figure 3.8B), a few A β plaques in hippocampal tissue from a patient with low OSA (Figure 3.8D), and more A β plaques in the brain of a patient with high OSA (Figure 3.6F). Some of the high OSA brains resembled AD in the extent of their A β burden (Figures 3.8F & H).

When stratified by CPAP use, no relationship was found between OSA severity and A β burden in CPAP users, whereas there was a significant positive relationship between OSA severity and A β burden in CPAP non-users (Figure 3.7D). In terms of correlation coefficient (r^2), the relationship between OSA severity and A β burden in CPAP non-users was stronger than the relationship between OSA severity and A β burden in the whole sample ($r^2=0.360$ vs. $r^2=0.134$). Further, the relationship between CPAP users was weaker than the relationship for the whole sample ($r^2=0.059$ vs. $r^2=0.134$).

The number of A β plaques in 5 different regions of the hippocampus was counted and related to OSA severity (Figure 3.10). No significant relationships were found in any of the regions.

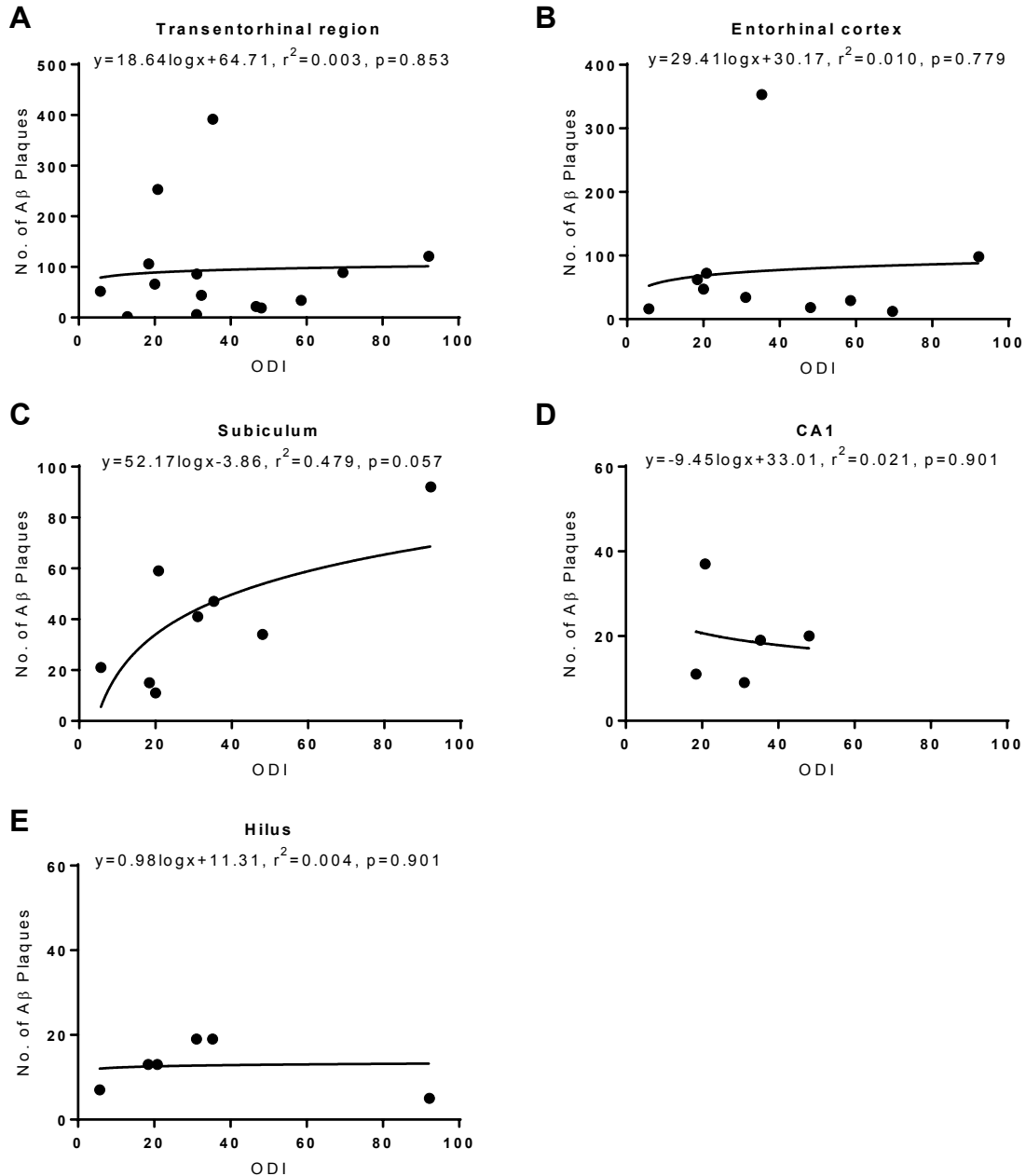


Figure 3.10 Regression between OSA severity (ODI) and A β plaque count in 5 regions of the hippocampus and surrounding cortex. Transentorhinal region (A), entorhinal cortex (B), subiculum (C), CA1 (D) and hilus (E).

3.3.5 AD pathology in the hippocampus as a function of age

It is well known that there is a relationship between AD pathology and age in AD patients; therefore this relationship was investigated in the context of OSA patients. Age was significantly related to NFT burden ($r^2=0.314$, $p=0.001$; Figure 3.11A), all patients above 65

years of age had some degree of NFT burden. Age was not related to A β burden ($r^2=0.019$, $p=0.432$; Figure 3.11B), there was an even spread of A β pathology burden across all ages.

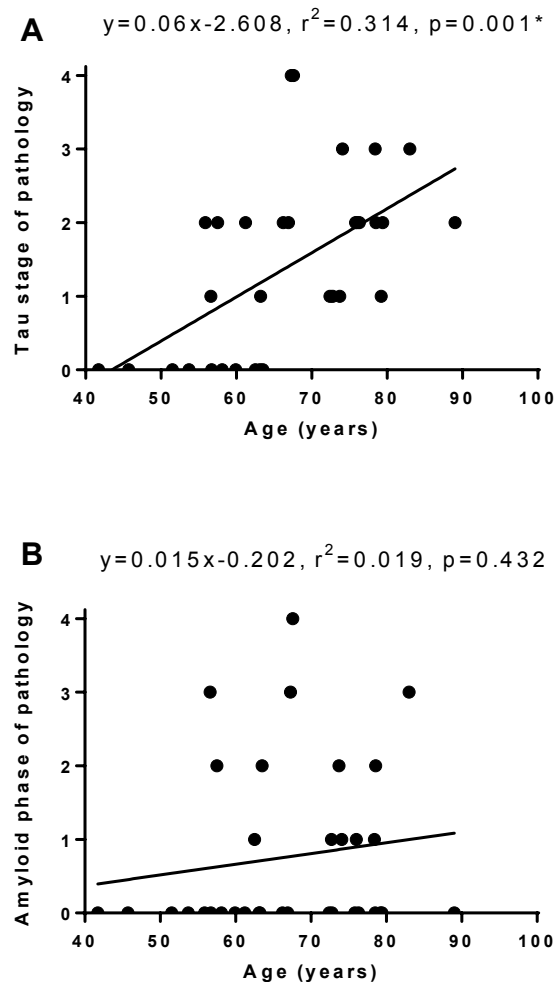


Figure 3.11 Relationship between NFTs and A β with age. Linear regression of NFT stage (A) and A β phase (B) with age in the total hippocampus sample.

3.3.6 AD pathology in the hippocampus compared with in the brainstem

A total of 22 brains had samples from both the hippocampus and the brainstem at the level of the pons. Of these, 16 brains had tau in the hippocampus and in the brainstem, and 15/16 brains had tau specifically in the LC.

A β plaque deposition was less consistent. Of the 22 brains that had samples from both locations, 9 had A β plaques in the hippocampus, yet only 2 of these also had A β plaques in the brainstem. A Fisher's Exact Test for independence found no significant difference between the distributions of pathology in the hippocampus and brainstem, $n=22$, $p=0.409$, suggesting that no relationship exists between the two.

3.4 Discussion

The present study investigated the extent of AD-like neuropathology in the hippocampus and brainstem of Icelandic OSA patients who had been diagnosed with different severities of OSA. As the severity of OSA increased, the NFT and A β burdens increased significantly in the hippocampus, but not in the brainstem. Age contributed to the relationship between OSA severity and NFT burden, but not to A β burden. Apo E genotypes were distributed as expected for the group and are not likely to have affected the results. CPAP treatment was found to be associated with less pathology burden, particularly for those with higher ODI values. These results may indicate that CPAP has the potential to delay the development of AD pathology.

Almost 90% of the pons sections examined contained some phosphorylated tau, whereas less than a quarter of the sections had A β pathology. As far as we are aware, no previous studies have investigated the presence of AD pathology in the brainstem of non-demented elderly persons. One study has estimated the prevalence of brainstem pathology in AD patients, and all patients in the sample were found to have NFTs in the raphe system, and 56% and 62% were found to have NFTs and A β plaques in the LC, respectively (Parvizi et al., 2001). The higher percentage of A β plaques found in that study compared to the present study is likely due to the participants in the Parvizi study having clinically-diagnosed AD, whereas patients in the present sample had not been diagnosed with any form of dementia. It is interesting that the present study reported a higher percentage of brains with NFTs in the LC (68%) than the previous study of AD patients (56%).

It is possible that the LC is more vulnerable to injury in OSA than it is in AD, since the noradrenergic neurons of the LC are wake-activated neurons that are most active during wakefulness, decrease their activation during NREM sleep and are completely inactive during

REM sleep (Murillo-Rodríguez et al., 2012). In animal models of IH (J. H. Zhang et al., 2010) and intermittent short sleep (Zhu et al., 2016) the LC has been shown to experience substantial neuronal loss. Neuronal loss was also seen in the LC of human brains with OSA (Gelber et al., 2015), however that study did not investigate the presence of NFTs or A β plaques in that nucleus. In OSA, LC neurons may be over-activated by the lack of continuous sleep, leading to mitochondrial oxidant stress and the phosphorylation of tau, making the development of NFTs more likely (Iijima-Ando et al., 2012; J. Zhang et al., 2014).

It has been suggested that in AD, pathological changes in the brainstem may precede changes in the hippocampus (Braak & Del Tredici, 2015; Braak et al., 2011). Conversely, there is evidence of simultaneous development of pathology in the LC and the entorhinal cortex in AD patients (Arendt, Bruckner, Morawski, Jager, & Gertz, 2015). While the present study found pathological changes in the brainstem in OSA patients, whether they developed before, after or simultaneously to hippocampal changes cannot be determined. All but one of the brains examined had tau in both locations. The extent of pathological accumulation in the brainstem was far less than that seen in the hippocampus, with most pons sections containing fewer than 20 NFTs, possibly indicating that these changes occur after hippocampal damage has begun. The distribution of A β was enlightening. Only two brains had A β at both locations, while 4 had A β only in the brainstem and 7 had A β only in the hippocampus. Furthermore, A β burdens were much higher in the hippocampus than in the LC. The lack of a correlation between these two sites suggests that A β deposition is unlikely to begin in the LC in OSA.

In the present study, 70% of patients had some degree of NFT burden and 38% had some degree of A β burden in their hippocampus, which is comparable to estimates made by Braak et al. (2011) that approximately 95% of elderly individuals had some degree of NFT burden and 35% had some degree of A β burden. These estimates were derived from a population with a narrower age range (61-70 years) than in the current study (41.7-89 years), which may account for the lower percentage of NFTs found here. Neither the OSA status nor the cognitive status of the patients were recorded for the Braak & Braak study, therefore their estimates are likely to include patients with OSA and with clinical symptoms of AD. Like the present study, Braak & Braak (2011) have reported that NFTs are more frequently observed than A β in early stages of AD, suggesting that NFT development precedes A β accumulation. The present study found that A β was rarely present by itself in the hippocampus, whereas

NFTs frequently were. Furthermore, in brains with a low NFT or A β burden, pathology was consistently limited to the region near the collateral sulcus, whereas in brains with heavier burdens of pathology, the distribution closely resembled those reported for AD (Braak et al., 2006; Thal et al., 2000). This pattern suggests that the spatiotemporal spread of pathogenesis is identical for the two disorders. However, it is unclear what aspect of OSA would cause the pathogenesis to begin in the collateral sulcus.

The present study has demonstrated that a relationship exists between OSA severity and the burden of NFTs and A β plaques in the hippocampus and nearby regions. However, while some patients may have had mild cognitive impairment or undiagnosed AD, none had symptoms that were salient enough to attract a diagnosis, even though some had a density of NFTs and A β plaques that was sufficient to qualify as AD. Furthermore, some patients with mild OSA had many A β plaques and/or tangles, whereas some patients with severe OSA and no recorded CPAP use had little or no neuropathology. Finally, one patient diagnosed with AD, was confirmed not to have had OSA (Fig. 3.8G & H). *Together these observations indicate that while untreated severe OSA tends to increase the burden of AD neuropathology, the presence of OSA is neither sufficient nor necessary to cause AD.* We speculate that the episodes of hypoxia, followed by re-oxygenation causes oxidative stress that injures the hippocampus and adjacent regions. Thus OSA renders these areas more vulnerable to the (as yet unknown) causative agent in AD, thereby facilitating the pathogenesis of this disease.

It has been shown in a mouse model that sleep is necessary for the clearance of A β from the brain (L. Xie et al., 2013). Interestingly, decreased NREM slow wave sleep is seen in both AD (Vitiello & Borson, 2001) and OSA (H. Kim et al., 2016; Yaouhi et al., 2009). Mander et al. (2015) suggest a self-perpetuating cycle may exist between A β deposition and decreased NREM slow wave sleep, where the deposition of A β is the start of the cycle. It may be that the lack of NREM sleep seen in OSA contributes to an inefficient clearance of A β from the brain. However, if this is the case, it does not account for the regional concentrations of A β that were observed in the present study.

Individual hippocampal regions were examined for pathology and the number of NFTs was related to ODI only in the CA1. CA1 is the most vulnerable hippocampal region to hypoxic injury (Duvernoy, Cattin, Risold, Vannson, & Gaudron, 2013). This region may become more susceptible to the development of AD pathology, as a result of hypoxic injury in OSA. Alternatively, this may simply reflect the Stage of NFT burden. In the current sample, NFT

pathology was most frequently categorised as Stage 2, and in AD, Stage 2 NFT pathology is characterised by the appearance of NFTs in the CA1 (Braak et al., 2006).

Individuals with Down syndrome (trisomy 21) frequently develop a progressive dementia in middle-age that is preceded by the deposition of A β plaques and NFTs in the cerebral cortex and hippocampal formation (Head & Schmitt, 2014). These degenerative changes have generally been attributed to the extra copy of chromosome 21 that contains a gene coding for amyloid precursor protein (Head & Schmitt, 2014). It is pertinent that the craniofacial structure in Down syndrome causes most individuals to suffer from OSA (Trois et al., 2009). The data from the present study raise the interesting possibility that OSA may accelerate the pathogenesis of AD-neuropathology in Down syndrome. This possibility could be tested by examining whether the onset of dementia in Down syndrome is affected by the severity of comorbid OSA.

While the presence of Apo E4 is a strong genetic risk factor for AD, the Apo E4 allele was not overrepresented in the 26 OSA patients that were successfully tested for Apo E status in the present study. These results concur with a previous meta-analysis that found no association between OSA and Apo E status (Varvarigou et al., 2011). Thus the present results suggest that OSA accelerates the pathogenesis of AD independently of Apo E status.

The present study has shown that regular CPAP use is associated with a shift in the relationship between OSA severity and the burden of AD pathology. In those who regularly used CPAP, there was no significant relationship between the burden of pathology and OSA severity, whereas in those who did not use CPAP, the relationship was highly significant. These results appear to indicate that the injury caused to the brain by OSA can be prevented or repaired, provided that CPAP is used diligently. This suggestion is supported by a report that in patients with moderate-severe OSA, three months of CPAP treatment increased the volume of the hippocampus and was accompanied by a modest improvement on tests of memory and executive function, although performance did not return to control levels (Canessa et al., 2011). As far as we are aware, *the present study is the first to demonstrate a significantly lower burden of AD-like neuropathology in response to a clinical intervention*, and it adds support to claims that CPAP may provide benefit in AD (Ancoli-Israel et al., 2008; Cooke, Ayalon, et al., 2009; Troussière et al., 2014). Those results, combined with findings from the present study, provide impetus for investigating whether regular CPAP use can reduce the burden of A β and NFTs in AD and in individuals with prodromal AD.

While several significant results were obtained in the present study, there are also limitations to be considered. Aside from the methodological limitations associated with this study sample (discussed further in Chapter 6: Discussion & Conclusions), the lack of tissue from other brain regions needs to be acknowledged. Typically AD is diagnosed from an investigation of tissue from the hippocampus and other neocortical regions, including the frontal, parietal and occipital lobes that develop AD pathology in the later stages of the disease (Braak et al., 2006; Thal et al., 2000). NFT pathogenesis is considered to follow a six-stage process, with no significant change in pathology in the hippocampus at Stages 5 and 6. These stages constitute increased pathology initially in high order association areas (Stage 5), and then in secondary and primary association areas (Stage 6). Stages 5 and 6 of NFT burden could not be determined in the present study, as tissue from cortical regions other than hippocampus was not available; therefore this study was not able to determine the stage of NFT pathology beyond Stage 4. A prospective study aiming to investigate the presence of AD pathology in OSA could plan to collect tissue from all of the necessary regions in order to overcome this limitation.

In conclusion, the present study has demonstrated that the burdens of NFTs and A β in the hippocampus increase with increasing OSA severity, and that this relationship is attenuated by the use of CPAP. Age plays a role in the relationship between OSA severity and NFT pathology, but not A β pathology. A relationship between the number of NFTs and ODI was found specifically in the CA1, the most vulnerable hippocampal region to hypoxia. The current findings are preliminary and it will be necessary to confirm these results in a larger sample. AD is a complex disorder and the present results clearly indicate that OSA is not the cause of AD; rather it appears to potentiate the pathogenesis of AD. OSA is a common disorder and is usually diagnosed decades before AD; interestingly the neuropathological signs of AD also appear decades before the clinical symptoms are detected (Braak et al., 2011). We speculate that untreated OSA may account for many of the instances of ‘cognitively normal’ individuals who have been found to have substantial A β burdens, and are currently considered to be at a prodromal stage of AD.

Chapter 4 Astrocyte and microglial neuropathology in the hippocampus of obstructive sleep apnoea patients

Obstructive sleep apnoea (OSA) involves intermittent hypoxic episodes throughout sleep, leading to excessive daytime sleepiness and cognitive impairment. Some symptoms (eg. tiredness, lack of energy) respond very quickly to treatment with CPAP, whereas other cognitive symptoms (eg. memory impairments) take much longer to recover. The delayed recovery may indicate that OSA injures the brain, and if so, it should be possible to detect this injury by the presence of reactive gliosis, which is part of the neuroinflammatory response. The present study investigated reactive gliosis and neuroinflammation in the hippocampus of OSA patients using quantitative immunohistochemistry. Increasing OSA severity was found to be significantly related to increased numbers of microglia in the hilus ($r^2=0.134$, $p=0.033$) and subiculum ($r^2=0.197$, $p=0.010$) and increased numbers of GFAP-positive astrocytes in the subiculum ($r^2=0.132$, $p=0.038$). An increased intensity of GFAP staining was seen in the entorhinal cortex ($r^2=0.178$, $p=0.028$) and CA3 for CPAP non-users only ($r^2=0.431$, $p=0.008$). A decreased intensity of S100b staining was seen in the CA3 ($r^2=0.123$, $p=0.042$). Most of these neuroinflammatory changes were found to be attenuated by CPAP use, with CPAP non-users having stronger relationships with markers of reactive gliosis than CPAP users. For the first time, neuropathological evidence is reported of reactive gliosis in the hippocampus of OSA patients, with implications for the beneficial effects of CPAP treatment.

4.1 Introduction

Obstructive sleep apnoea is an increasingly prevalent health concern with estimates of prevalence ranging from 3-10% in adults younger than 40 years of age to 10-20% in adults older than 65 (Gislason, 2014; Punjabi, 2008; Simpson et al., 2013). A recent review suggested the prevalence of moderate-severe OSA may be as high as 36% in adults over 60 years of age (Senaratna et al., 2017). OSA involves temporary cessations in breathing during sleep, resulting in transient arterial oxygen desaturations and frequent arousals from sleep. Patients with OSA often experience cognitive symptoms that include impairments in memory, attention and executive functioning (Ferini-Strambi et al., 2003; Torelli et al., 2011). CPAP is an effective treatment for OSA (Buchanan & Grunstein, 2011), and quickly alleviates some of the cognitive symptoms, particularly in patients with mild-moderate OSA.

However, patients with severe OSA tend to have more extensive and more persistent deficits (Canessa et al., 2011; Lau et al., 2010), suggesting that severe OSA may damage the brain. Several lines of evidence support this conclusion, and indicate that severe OSA is associated with an increased neuroinflammatory response. For instance, increased levels of pro-inflammatory cytokines are present in the blood of OSA patients (Alberti et al., 2003; de la Peña Bravo et al., 2007), and some of these are specific to the brain, indicating the presence of neuroinflammation. Specifically, increases in the astrocyte marker S100b have been found in the blood of OSA patients when compared to controls (Braga et al., 2006; Duru et al., 2012). S100b is a marker of reactive gliosis, a key component of the neuroinflammatory response to injury. Further, MRS imaging has found increased levels of myo-inositol, a marker for reactive gliosis, in the frontal, temporal and insular cortices of OSA patients compared to controls (Sarchielli et al., 2008; Sharma et al., 2010; Yadav et al., 2014).

Glial cells are vitally important for maintaining homeostasis in the microenvironment of the brain. Astrocytes provide essential nutrients and metabolites to neurons, maintain the integrity of the BBB, provide antioxidant support, and respond to injury and inflammation (Steele & Robinson, 2012). Microglia are the immune cells of the brain, and they are specialised to react to injury signals. 'Reactive gliosis' is the injury response state of glia during which they increase in numbers and/or size and undergo morphological changes. While both astrocytes and microglia can undergo gliosis, microglia appear to be more sensitive and they respond first to brain injury (D. Zhang, Hu, Qian, O'Callaghan, & Hong, 2010). Reactive microgliosis consists of an increase in cell numbers at the injury site, increased phagocytosis, an upregulation of proinflammatory cytokines and morphological changes. The morphology of microglia transitions from a resting state in which cells have a stellate appearance with many processes, to a reactive state in which cells retract most of their processes and assume an 'amoeboid' shape with just a few processes that allows them to migrate through the interstitial space (Aloisi, 2001; Block & Hong, 2005). Macrophage antigen complex-1 (Mac-1) also called Complement receptor 3 (CR3) is a microglial membrane receptor that consists of the proteins CD11b and CD18. Mac-1 is important for phagocytosis and its expression is increased in neurodegeneration (Block, Zecca, & Hong, 2007). Reactive microgliosis occurs after ischaemic injury in both humans and animal models (Lambertsen, Biber, & Finsen, 2012; Weinstein, Koerner, & Mller, 2010). Significantly, reactive microgliosis has also been found in brains exposed to IH (Sapin et al., 2015; Smith et al., 2013). Increased microglial inflammatory markers were detected in the hippocampus and

cerebral cortex after 2, 6 and 24 weeks exposure to IH (Sapin et al., 2015; Smith et al., 2013). Additionally, Sapin and colleagues (2015) noted significant morphological changes, including decreased ramification and thickening of primary processes as well as an increased numerical density of microglial cells; features that are all characteristic of reactive microgliosis. Titres of inflammatory markers, including cytokines known to be released by microglia, are increased in the serum of OSA patients compared to controls (Alberti et al., 2003; de la Peña Bravo et al., 2007; Nadeem et al., 2013). However, as many of these markers are also increased in systemic inflammatory conditions which are common comorbidities of OSA, it is not yet known if microgliosis is present in the brains of OSA patients.

Astrocytes react to neuroinflammation and injury by proliferating, altering their expression of numerous astrocyte-specific proteins and by changing their morphology (Eddleston & Mucke, 1993; Sofroniew, 2009). Unlike the amoeboid changes seen in reactive microglia, astrocytes display hypertrophy in response to tissue injury, and their processes tend to become thicker and more prominent (D. Sun & Jakobs, 2012). Reactive gliosis can be neuroprotective or neurotoxic, depending on injury type and duration. Acute reactive gliosis in response to a localised tissue injury is protective (Myer, Gurkoff, Lee, Hovda, & Sofroniew, 2006), but chronic reactive gliosis can be detrimental (Wilhelmsson et al., 2004). In AD, reactive astrocytes are associated with A β plaques (Nagele et al., 2003; Nagele et al., 2004), and interventions that reduce the extent of reactive gliosis are associated with accelerated A β plaque development (Kraft et al., 2013), highlighting a protective role for astrocytes. However, chronic reactive gliosis that is not associated with a specific injury site can impair learning and memory (Lokensgard et al., 2015). For instance, chronic reactive gliosis induced by an ischaemic injury impairs hippocampal-dependent learning and memory (Evonuk, Prabhu, Young, & DeSilva, 2017). The hippocampus is highly vulnerable to hypoxic injury, and given that OSA is a chronic disease, reactive gliosis may be present at this location.

Many glial proteins are up- or down-regulated during reactive astrogliosis (Eddleston & Mucke, 1993; Sofroniew, 2009) and the profile of protein expression has been found to differ by injury type (Anderson et al., 2014; Zamanian et al., 2012). Zamanian et al. (2012) reported variations in astrocytic protein regulation between animals exposed to ischaemic stroke or neuroinflammation (caused by intraperitoneal injection of bacterial endotoxin). While there was overlap between the gene alterations, at least 50% of the changes were unique to the

specific injury. GFAP expression was increased in both injury types (Zamanian et al., 2012). Further, astrogliosis is a graded response, so the extent of the morphological and functional changes depends on the strength of the stimulus (Anderson et al., 2014). These findings highlight the need to use a variety of markers to investigate reactive astrogliosis; relevant astrocyte markers for this project are GFAP, S100b and GS.

GFAP is a cytoskeletal intermediate filament protein involved in maintaining the structure of astrocytes; in the brain its expression is almost exclusively restricted to astrocytes. The upregulation of GFAP expression has long been used to identify reactive astrocytes. The specific functions of GFAP are currently unknown; GFAP-null mice show no significant deficits compared to wild-type mice, except after CNS injury (De Mattos Coelho-Aguiar et al., 2015). Mice deficient in GFAP and Vimentin (another intermediate filament protein) have poorer injury responses, including deficits in glial scar formation (Pekny et al., 1999). GFAP-null mice are particularly susceptible to ischaemic injury, suggesting a role for GFAP in vascular maintenance and BBB integrity (Brenner, 2014; Nawashiro, Brenner, Fukui, Shima, & Hallenbeck, 2000). GFAP expression is upregulated in both ischaemic brain injury and AD (Griffin et al., 1989; Tanaka, Araki, & Masuzawa, 1992; Verkhratsky, Olabarria, Noristani, Yeh, & Rodriguez, 2010). Increases in the number and size of astrocytes, as well as an upregulation of GFAP in individual cells, is evident in the hippocampus of animals exposed to ischaemic injury (Pivneva, Tsupikov, Pilipenko, Vasilenko, & Skibo, 2005; Romanini et al., 2015), particularly in the hilus and the CA1 regions (Pivneva et al.). Similarly the numbers of GFAP-positive astrocytes in the hippocampus are increased after exposure to IH for 10 days (Aviles-Reyes et al., 2010). Further, an increase in GFAP immunoreactivity and changes in astrocyte morphology are seen after 5 weeks of IH exposure (Baronio et al., 2013). Despite these findings from animal models, it is not known whether reactive astrogliosis or changes in GFAP expression occur in the brain in patients with OSA.

While various cells in the body express S100b, its expression in the brain is largely restricted to astrocytes. S100b is a soluble calcium-binding protein with multiple functions, including a role in the proliferation and migration of astrocytes during development, as well as being part of the neuroinflammatory response to injury (Donato et al., 2009). S100b is secreted by astrocytes as a neuroprotective factor, its expression and release from astrocytes is increased after brain injury, including ischaemia and AD (Griffin et al., 1989; Matsui et al., 2002). Extracellular S100b stimulates the release of proinflammatory cytokines from astrocytes and

microglia, promoting further reactive astrogliosis and intracellular S100b expression (Donato et al., 2009; Matsui et al., 2002). Increased S100b production by astrocytes has been detected in rodents exposed to IH, specifically in the cerebellum (Baronio et al., 2013), cerebral cortex and hippocampus (Aviles-Reyes et al., 2010). Aviles-Reyes et al. (2010) found that 10 days of exposure to IH leads to increased S100b expression and neuroprotection. Interestingly, however, the same group found that blocking S100b expression did not worsen neuronal death (Angelo et al., 2014). Additionally, overexpression of S100b leads to increased reactive gliosis, neuronal death and increased amyloid deposition (Angelo et al., 2014; Mori et al., 2010). It has been suggested that S100b is neuroprotective at low concentrations, activating cell survival pathways; whereas high concentrations are neurotoxic by activating apoptotic pathways (Donato et al., 2009). It is not yet known whether S100b levels are altered in the brains of patients with OSA. As previously mentioned, OSA patients have increased serum levels of S100b (Braga et al., 2006; Duru et al., 2012), however it is not known with certainty whether these increases reflect a state of reactive gliosis in the brain.

GS is expressed by most cells in the majority of mammalian tissues, yet in the brain, GS expression is largely limited to astrocytes. GS is the enzyme necessary for the amidation of neurotoxic glutamate to form glutamine. Glutamine is then transported to neurons for deamidation into glutamate. The enzymatic activity of GS is sensitive to inactivation by oxidative stress (Fernandes, Dringen, Lawen, & Robinson, 2011; Robinson, Lee, Bishop, Czerwinska, & Dringen, 2015), and decreased GS expression in astrocytes has been detected in many central nervous system diseases (C. F. Rose, Verkhratsky, & Parpura, 2013). In AD for instance, GS immunoreactivity is reduced in astrocytes in the inferior temporal lobe, a region adjacent to the hippocampus (Robinson, 2000, 2001). Hypoxic injury in animals is also associated with decreased GS expression in the hippocampus, cerebral cortex and cerebellum (A. Lee et al., 2010; Swamy, Salleh, Sirajudeen, Yusof, & Chandran, 2010). Specifically, GS loss was found in the CA1 of the hippocampus, but not in the dentate gyrus. Conversely, a mild and transient increase in GS expression was observed in the hippocampal hilus of gerbils following experimental hypoxic-ischaemic injury (Tanaka et al., 1992). The expression of GS has not been investigated in animal models of IH or directly in OSA patients, however, MRS imaging has found an increased glutamate signal in the insular cortex of OSA patients compared to controls (Macey et al., 2016). As GS is responsible for converting glutamate to glutamine, the increase in glutamate might be attributable to reduced activity of GS. An increase in the combined glutamate/glutamine level has been found in the

temporal and frontal lobes of OSA patients (Sharma et al., 2010), however the individual contributions of glutamate and glutamine to this effect have not been determined.

Currently, very little is known about reactive gliosis in OSA, despite the fact that it can serve as an important indicator of the extent of neuronal injury caused by OSA. A neuropathology study of the LC in autopsied brainstems from OSA patients found a correlation between nadir arterial oxygen desaturation levels and the severity of gliosis (Gelber et al., 2015). This finding suggests that severe OSA stimulates reactive gliosis. However, Gelber's study did not consider the individual effects of microgliosis and astrogliosis; indeed, glia-specific markers were not used, only general histological methods. Further, as Gelber's study did not examine forebrain regions, it is not possible to know whether their findings from a single brainstem nucleus reflect a general change throughout the brain. In order to gain a more comprehensive understanding, the present study has examined autopsy material from the hippocampus of patients with clinically-verified OSA, to determine whether OSA severity is associated with reactive microgliosis or astrogliosis, and if so, whether the extent of gliosis is reduced by CPAP treatment.

4.2 Methods

4.2.1 Subjects/samples

The study sample consisted of autopsy brain tissue from 34 patients in the Iceland cohort described in Chapter 2. Six cases were excluded from entorhinal cortex analysis due to insufficient tissue being available, but 34 cases were included for all hippocampal regions.

4.2.2 Tissue processing

Brain tissue that had been embedded in paraffin wax blocks was sectioned at 20µm on a microtome and then processed for histology or immunohistochemistry, as described in Chapter 2.

4.2.3 Immunohistochemistry

Immunohistochemistry was performed using CD11b to visualise microglial cells, while GFAP, S100b and GS were used to visualise astrocytes. The immunohistochemistry protocol followed previously described methods (Robinson, 2001). Briefly, after deparaffinisation,

sections to be immunostained for GFAP, S100b and CD11b were incubated for 30 min in target retrieval solution to expose antigenic sites; sections to be immunostained for GS required no target retrieval. Sections were incubated with blocking solution (PBS, 0.1 g BSA, 10% Triton X-100 and 1% ethanolamine) for 3 h at room temperature, followed by primary antibody solutions for 18 h at room temperature. Primary antibody concentrations were as follows: anti-GFAP (DAKO Z0334) was diluted at 1:20000, anti-GS (Sigma G2781) was diluted at 1:2500, anti-S100b (Abcam ab52642) was diluted at 1:5000 and anti-CD11b (Merck MABT149) was diluted at 1:1000. Sections were subsequently incubated for 4 h with appropriate secondary antibodies diluted at 1:300 and processed as described in Chapter 2.

All brain sections were processed in an identical manner. The examiner was blinded to the OSA severity of the patients when performing immunohistochemistry, counting cells and calculating intensity measures. Two hippocampus sections were stained for each brain; all cell counts and immunolabelling intensity measures are expressed as the mean of the two sections.

4.2.4 Glial cell counts

Cell counts were performed using Olympus CellSens software. Micrographs were taken in the hilus (CA4), CA3, CA1, subiculum and entorhinal cortex of the hippocampus at 400X magnification for microglia and 200X magnification for astrocytes (Figure 4.1). Anatomical landmarks were used to identify the location at which micrographs were taken. For example, the micrograph in the hilus was taken between the blades of the dentate gyrus. Micrographs were converted to grayscale and auto-contrasted to distinguish the cells from the background. Settings of the 'Count and Measure' feature of the CellSens program were then adjusted to achieve the most accurate cell count as determined by the examiner's visual inspection. An example of this can be seen in Figure 4.2. Any immunostained object smaller than 100 pixels in diameter in GFAP, S100b and GS images was excluded from the cell count. Any object smaller than 80 pixels in diameter in CD11b images was excluded from the cell count. Any object that was clearly identifiable as an astrocyte or microglial cell but not selected by the 'Count and Measure' feature was manually selected by the examiner. Likewise any object that was clearly not an astrocyte or microglial cell that was selected by the 'Count and Measure' feature was manually excluded by the examiner. This procedure was necessary as some blood vessels became prominent in the images after auto-contrasting and were

automatically selected. Also, in S100b images, some neurons displayed S100b staining and were selected as objects but were subsequently excluded by the examiner.

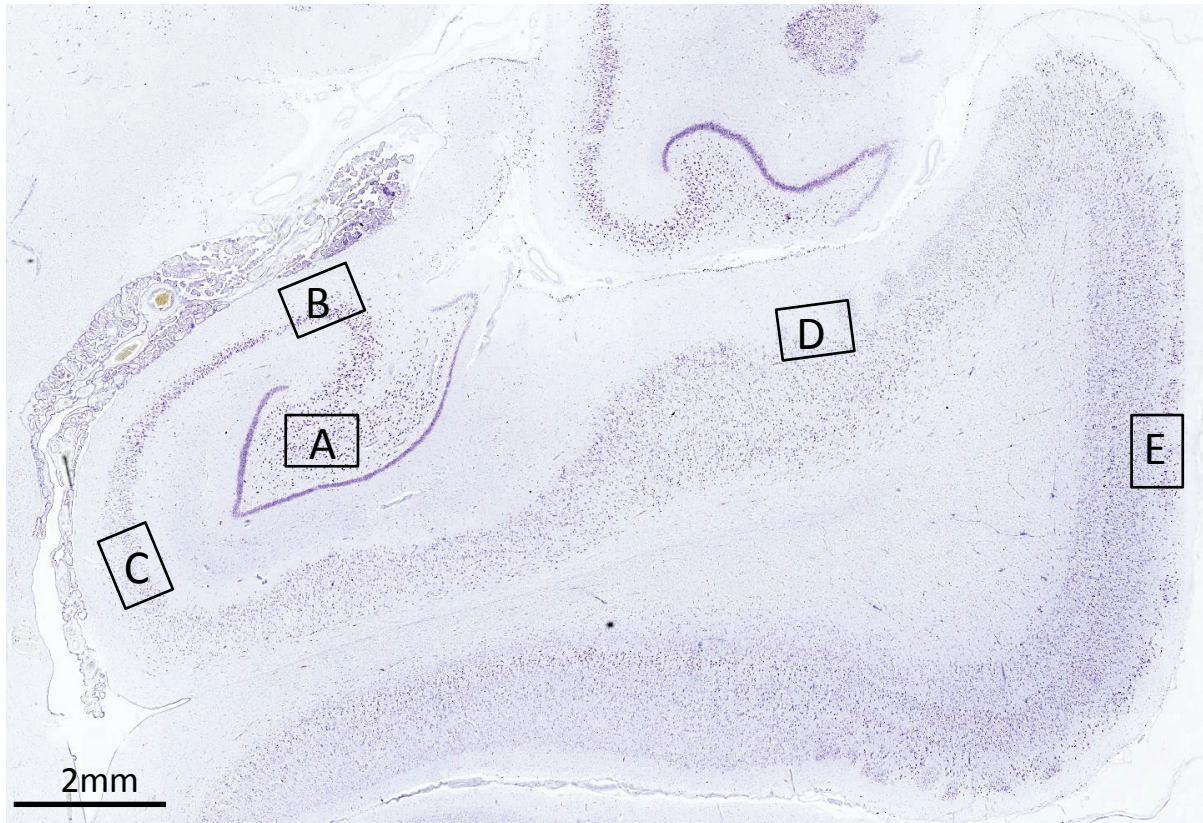


Figure 4.1 Low magnification image of hippocampus and entorhinal cortex. The regions examined for reactive gliosis were the hilus (A), CA3 (B), CA1 (C), subiculum (D) and entorhinal cortex (E).

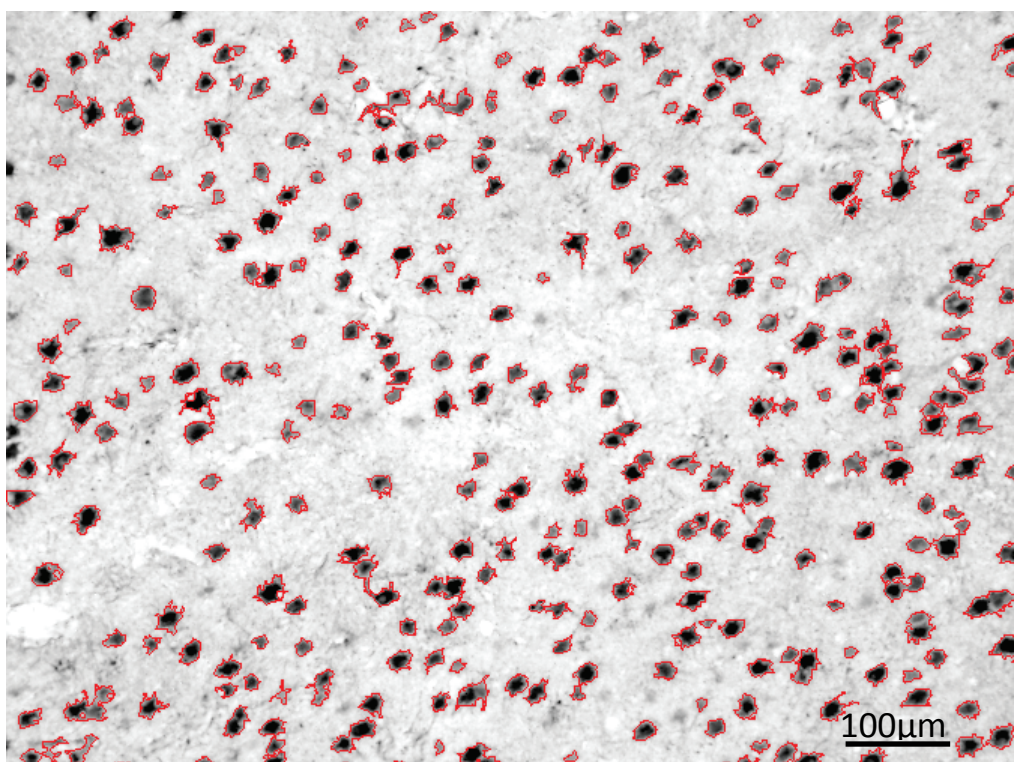


Figure 4.2 Example of cell counting procedure using CellSens. The image shows astrocytes stained with GFAP in the hilus. The image was converted to greyscale and auto-contrasted prior to cell counting. Cells selected for counting are encircled in red. Cell bodies that were bisected by the left hand or top margins of the micrograph were not included in the counts.

4.2.5 Glial staining intensity

Increases in the intensity of staining were determined using the same software and images as for the glial cell counts, except that the images were not auto-contrasted. The number of cells above a predetermined greyscale threshold were counted. The grey threshold was different for each antibody and was used as a semi-quantitative measure of upregulated expression of the glial markers. The number of cells above the grey threshold was expressed as a percentage of the total cell count previously obtained (see section 4.2.4). Object exclusion based on size was performed in the same manner as for the cell counts. No manual object inclusion was performed for staining intensity, as the purpose of this measure was to exclude objects below the specified threshold.

4.2.6 Microglia morphology

Microglial morphology was assessed using the images taken for the cell count analysis. Images were not converted to grayscale, and contrast adjustments were made in order to reveal more morphological detail. The morphology of microglial cells was assessed semi-quantitatively using a 5 point scale that estimated the proportion of cells that were in the resting, activated and amoeboid states. Level 1 of the 5 point scale corresponded to all microglia being in a resting state, level 2 consisted of mostly resting microglia (~75%) and some activated or amoeboid cells (~25%). Level 3 contained equal proportions of resting (50%) and activated or amoeboid microglia. Level 4 consisted of some resting microglia (~25%) but mostly activated or amoeboid cells (~75%), level 5 consisted of all activated or amoeboid microglia. Each image was given a score between 1 and 5 and the score was averaged across the two images from each brain, with higher values indicating more activated/amoeboid morphology and lower scores indicating more resting morphology.

4.2.7 Statistical analysis

All statistical analysis was performed using the IBM SPSS. Linear regressions between ODI as a marker of OSA severity and the various glia cell variables were performed. Similarly, linear regressions were performed when the sample was stratified by CPAP use.

4.3 Results

4.3.1 Descriptive statistics

Descriptive statistics for the samples are provided in Table 4.1. A significant correlation was found between age and ODI, $r=0.416$, $p=0.014$. No other significant correlations were found between age, ODI, BMI or interval from diagnosis to death. Differences between the CPAP users and non-users were analysed using two-tailed student t-tests and it was found that CPAP users had a significantly higher BMI than CPAP non-users, $t(27)=3.50$, $p=0.002$. No differences were found for age, ODI or interval from diagnosis to death.

Table 4.1 Descriptive statistics of the total sample and when separated by CPAP use. Mean \pm standard deviation.

	Total	CPAP users	CPAP non-users	Significance CPAP users vs. non-users
n	34	18	16	
Gender	F=18, M=16	F=12, M=6	F=6, M=10	
Age at death (years)	67.0 \pm 11.1	69.9 \pm 11.1	63.8 \pm 10.5	p=0.111
BMI kg/m²	29.5 \pm 6.2	32.6 \pm 5.8	25.7 \pm 4.4	p=0.002*
	n=29	n=16	n=13	
Time from OSA diagnosis to death (years)	7.3 \pm 5.8	7.1 \pm 6.2	7.5 \pm 5.7	p=0.815
ODI (events/h)	25.5 \pm 21.3	32.0 \pm 18.8	18.2 \pm 22.0	p=0.057

*p<0.05 CPAP users compared to CPAP non-users

4.3.2 Astrocyte and microglia cell counts

4.3.2.1 Astrocyte and microglia cell counts in the hilus (CA4)

In the hilus no significant relationships were found between cell count and ODI for any of the astrocyte markers (Figure 4.3A, B & C). Even when stratified by CPAP use, no significant relationships were found between ODI and any of the astrocyte markers (Figure 4.4A, B & C). Despite S100b being found predominantly in astrocytes, some hilus sections showed S100b-positive neurons as well (Figure 4.5). Nine of the 34 brains had S100b-positive neurons in the hilus of the hippocampus. However there appeared to be no relationship between the presence of S100b-positive neurons and ODI, or any of the other variables assessed in this study.

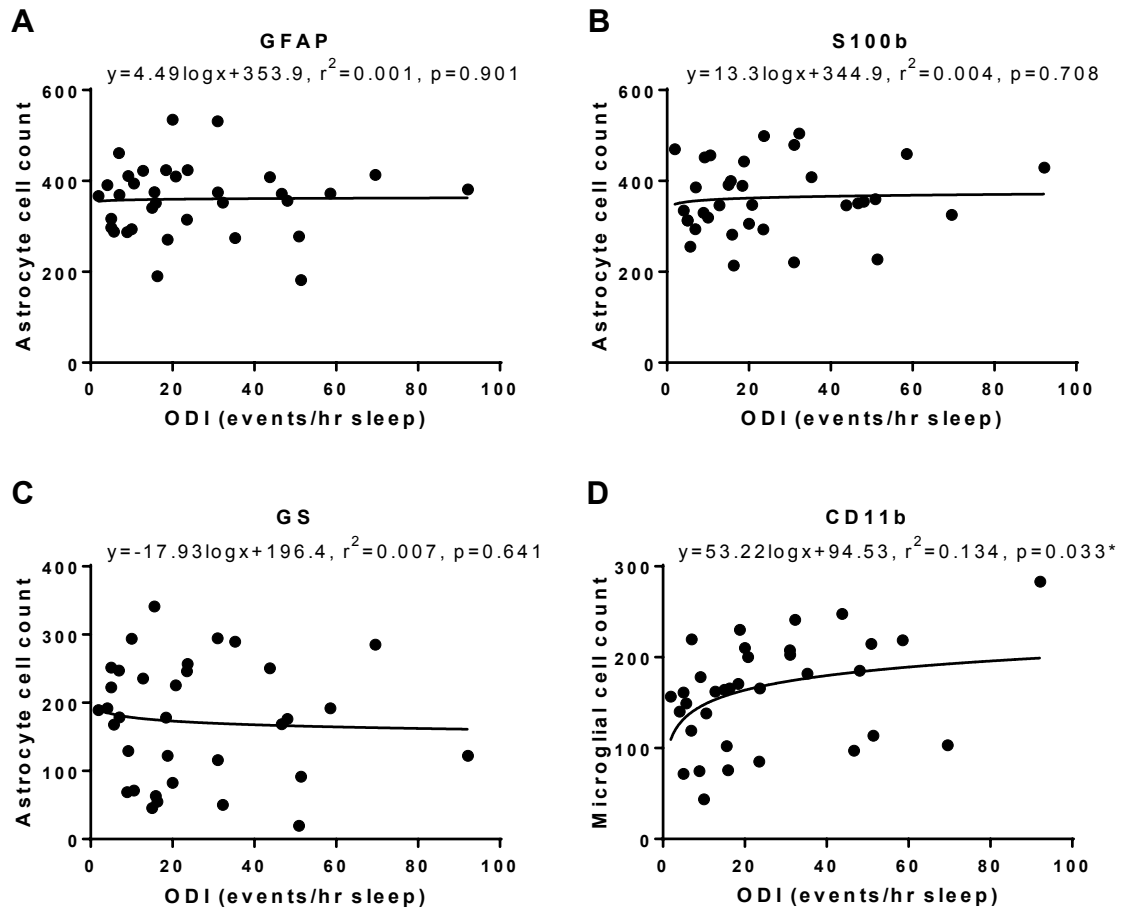


Figure 4.3 Glial cell counts in the hilus regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D). * indicates $p < 0.05$.

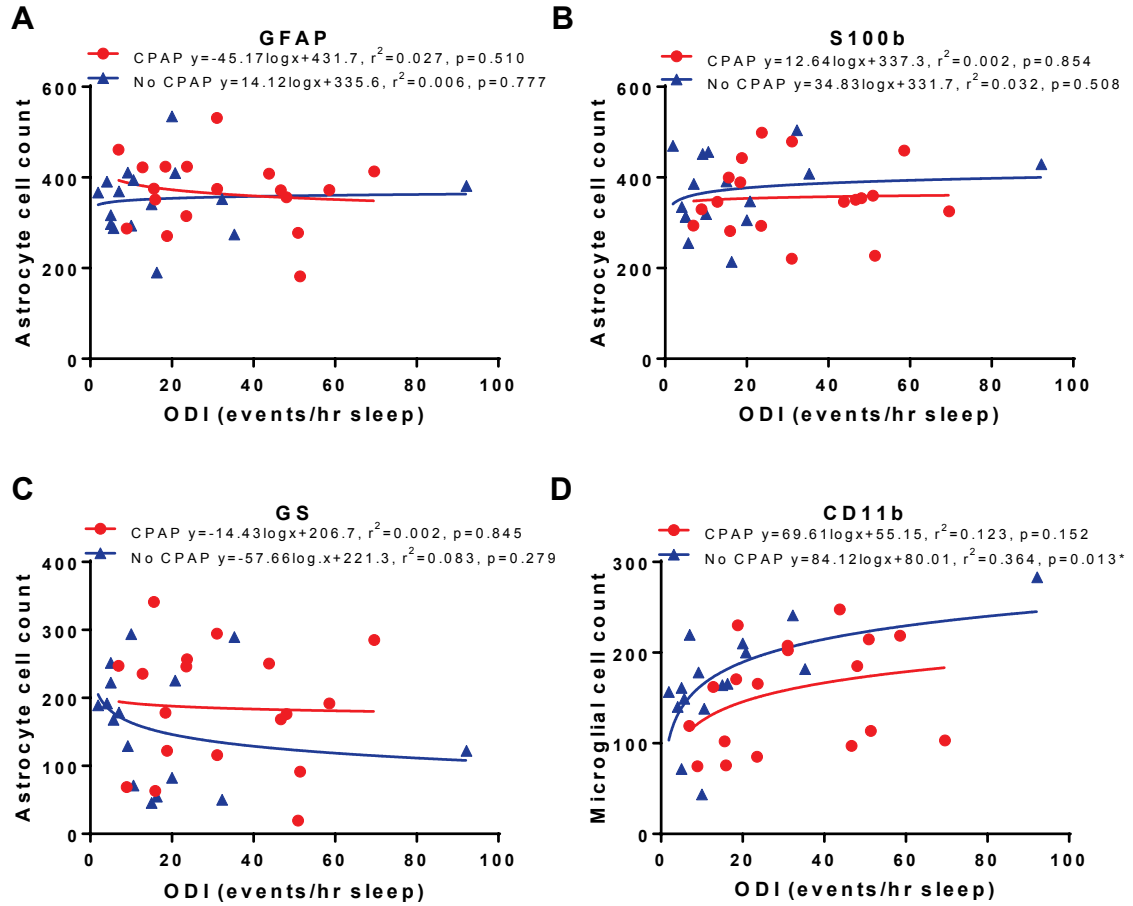


Figure 4.4 Glial cell counts in the hilus regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p < 0.05$.

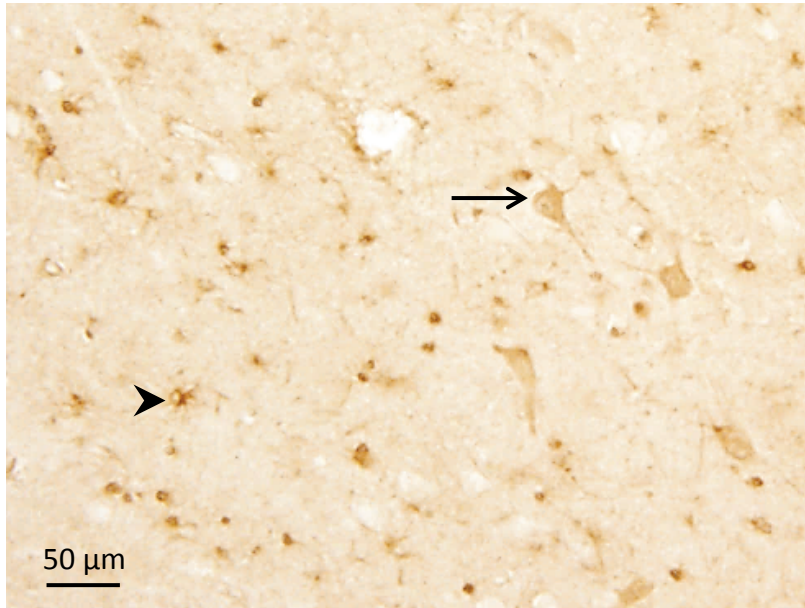


Figure 4.5 S100b-positive astrocytes and neurons in the hilus. Micrograph of S100b immunostaining in both astrocytes (arrowhead) and neurons (arrow) in the hilus.

In contrast, a significant positive relationship was found between the number of CD11b-positive cells and ODI, $r^2 = 0.134$, $p = 0.033$ (Figure 4.3D), indicating that microglial numbers increase with increasing ODI. Microglial morphology also changed with OSA severity. In a patient with low ODI, the microglia appear in their resting state (Figure 4.6A) and have a stellate appearance with long, thin processes (inset of 4.6A), whereas in a patient with high ODI, microglia appear in their reactive state (Figure 4.6B) and have larger cell bodies with fewer, thicker processes (inset of 4.6B). When the sample was stratified by CPAP use (Figure 4.4A), the relationship between ODI and CD11b-positive cells became stronger for CPAP non-users, $r^2 = 0.364$, $p = 0.013$, and weaker for CPAP users, $r^2 = 0.123$, $p = 0.152$. This pattern shows that CPAP non-users with a high ODI have more CD11b-positive microglial cells in the hilus than CPAP users.

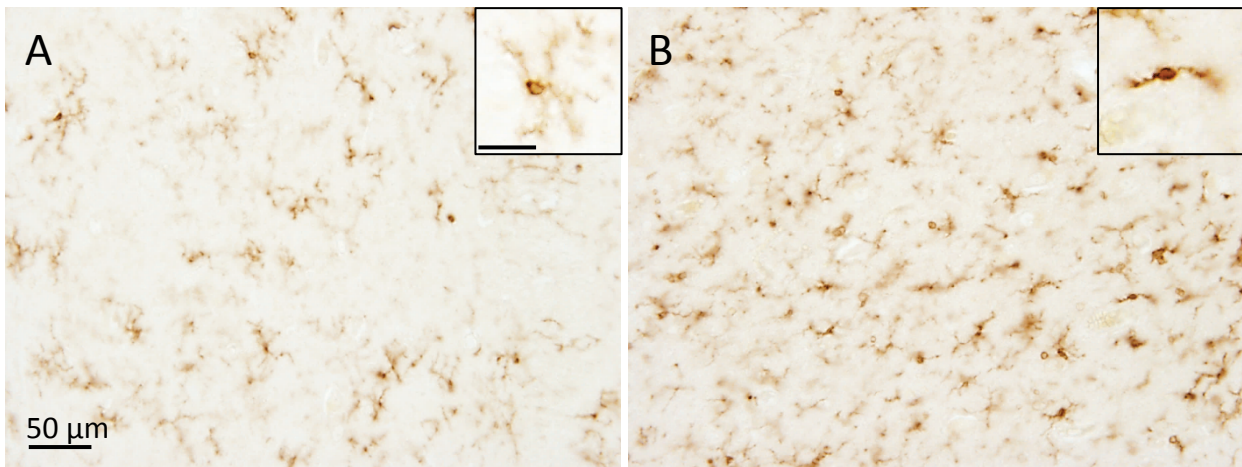


Figure 4.6 Microglia in the hilus. Micrographs of CD11b-positive microglia in the hilus from a patient with low OSA (A) and high OSA (B). Notice the increased number of cells in the right image compared to the left. The inset in A shows a resting microglial cell with many processes and the inset in B shows a reactive microglial cell with fewer processes and round dense cell body. Scale bar in A applies to both panels. Scale bar of inset = 20 µm and applies to both panel insets.

4.3.2.2 Astrocyte and microglia cell counts in the CA3

In the CA3 region there were no significant relationships between ODI and any of the cell counts for astrocytes or microglia (Figure 4.7). No relationships emerged when the sample was stratified by CPAP use (Figure 4.8).

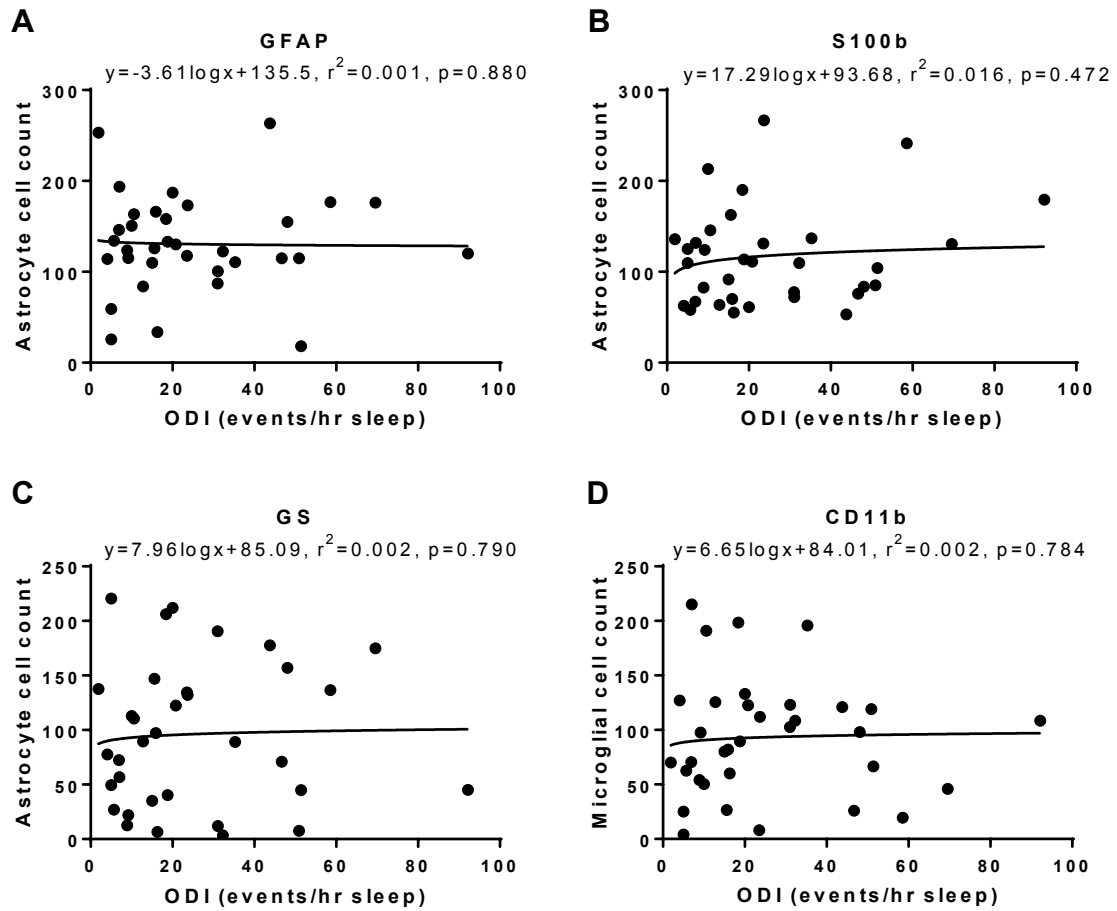


Figure 4.7 Glial cell counts in CA3 regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D). * indicates $p < 0.05$.

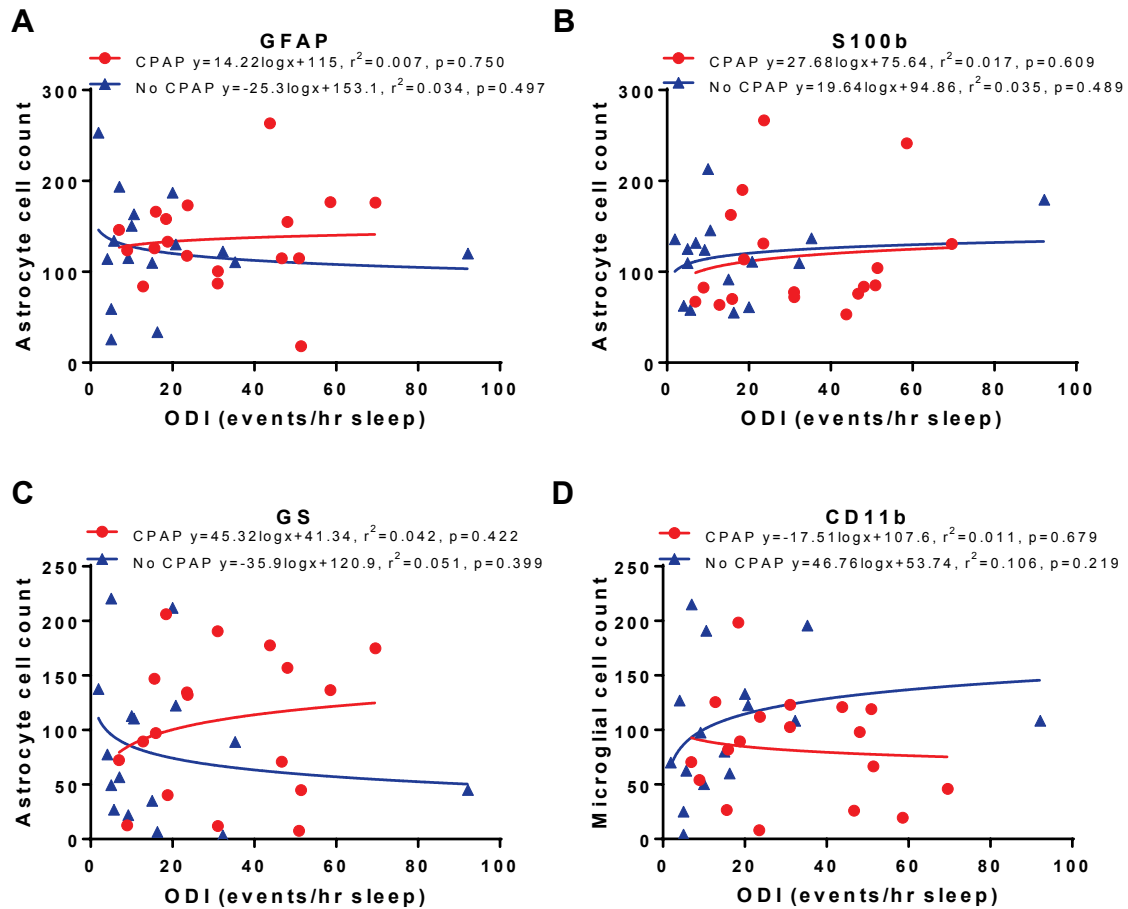


Figure 4.8 Glial cell counts in CA3 regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p < 0.05$.

4.3.2.3 Astrocyte and microglia cell counts in the CA1

In CA1, there were no significant relationships between ODI and the number of astrocytes or microglial cells (Figure 4.9). When stratified by CPAP use no significant relationships were seen (Figure 4.10).

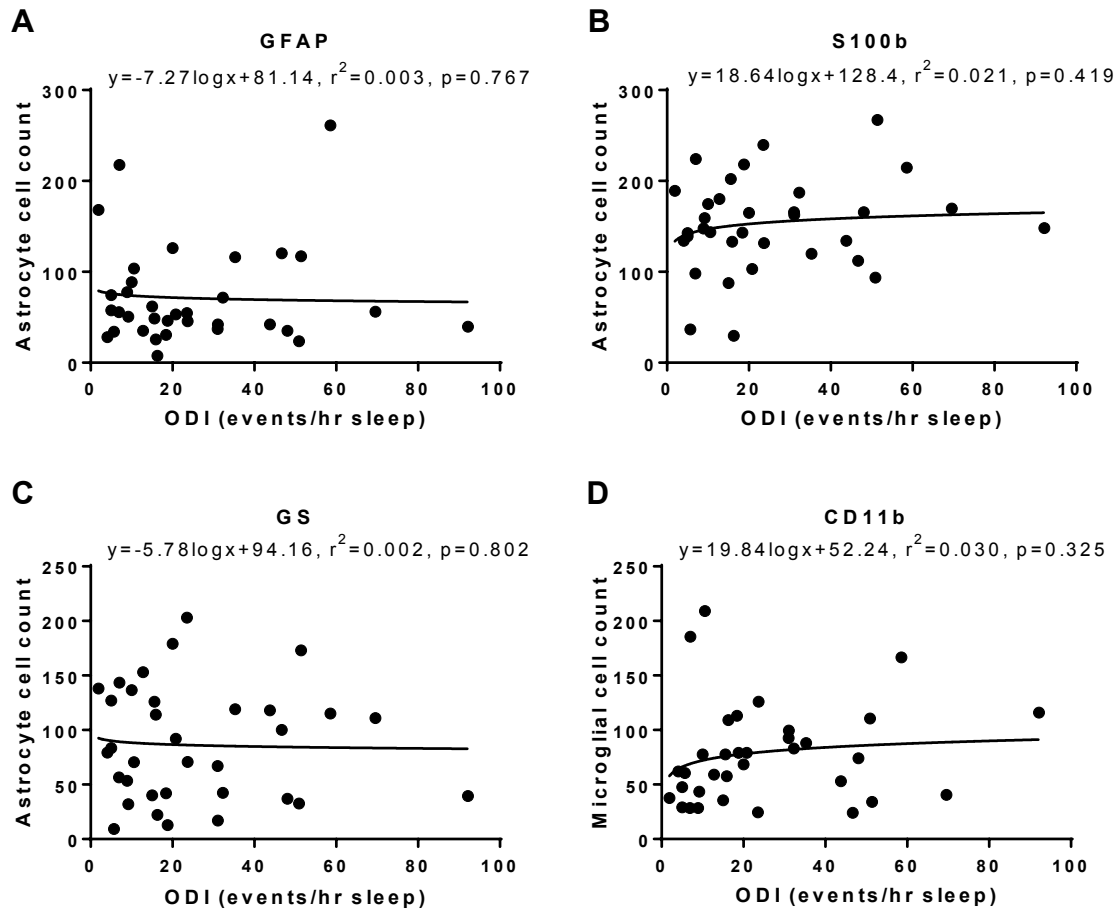


Figure 4.9 Glial cell counts in CA1 regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D). * indicates $p < 0.05$.

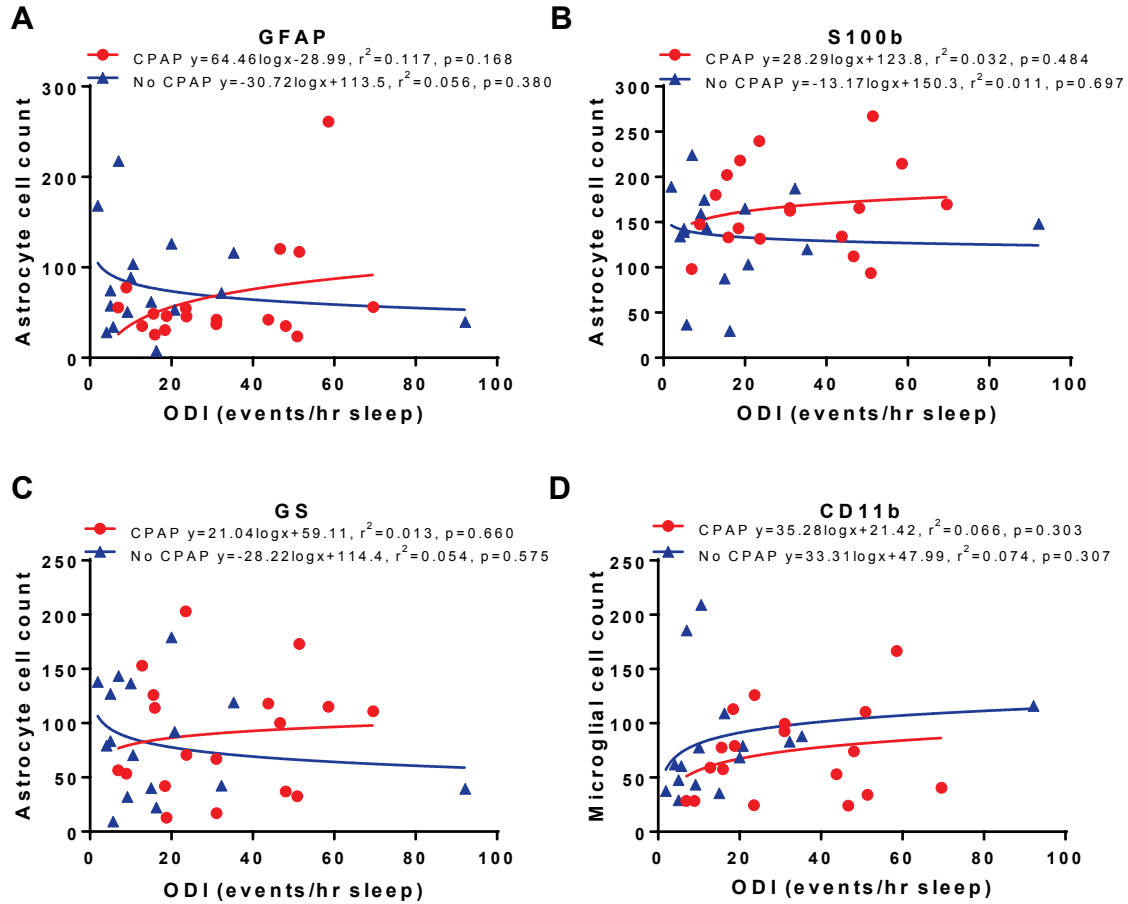


Figure 4.10 Glial cell counts in CA1 regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p < 0.05$.

4.3.2.4 Astrocyte and microglia cell counts in the subiculum

In the subiculum, no significant relationships were seen between ODI and S100b or GS. However, a significant positive relationship was found between ODI and the number of GFAP-positive cells, $r^2 = 0.132$, $p = 0.038$, (Figure 4.11A). The number of GFAP labelled astrocytes increased with increasing ODI (Figure 4.13). When CPAP users and non-users were considered separately, no significant relationships between ODI and GFAP-positive astrocyte cell count were found for either group (Figure 4.12A). This pattern suggests that there may be no effect of CPAP use on the number of GFAP-positive astrocytes in the subiculum.

In the subiculum, a significant positive relationship was also seen between ODI and the number of CD11b-positive microglia, $r^2=0.197$, $p=0.010$ (Figure 4.11D & Figure 4.14). This relationship was stronger for CPAP non-users, $r^2=0.487$, $p=0.004$, and was weaker and non-significant for CPAP users, $r^2=0.087$, $p=0.236$, (Figure 4.12D).

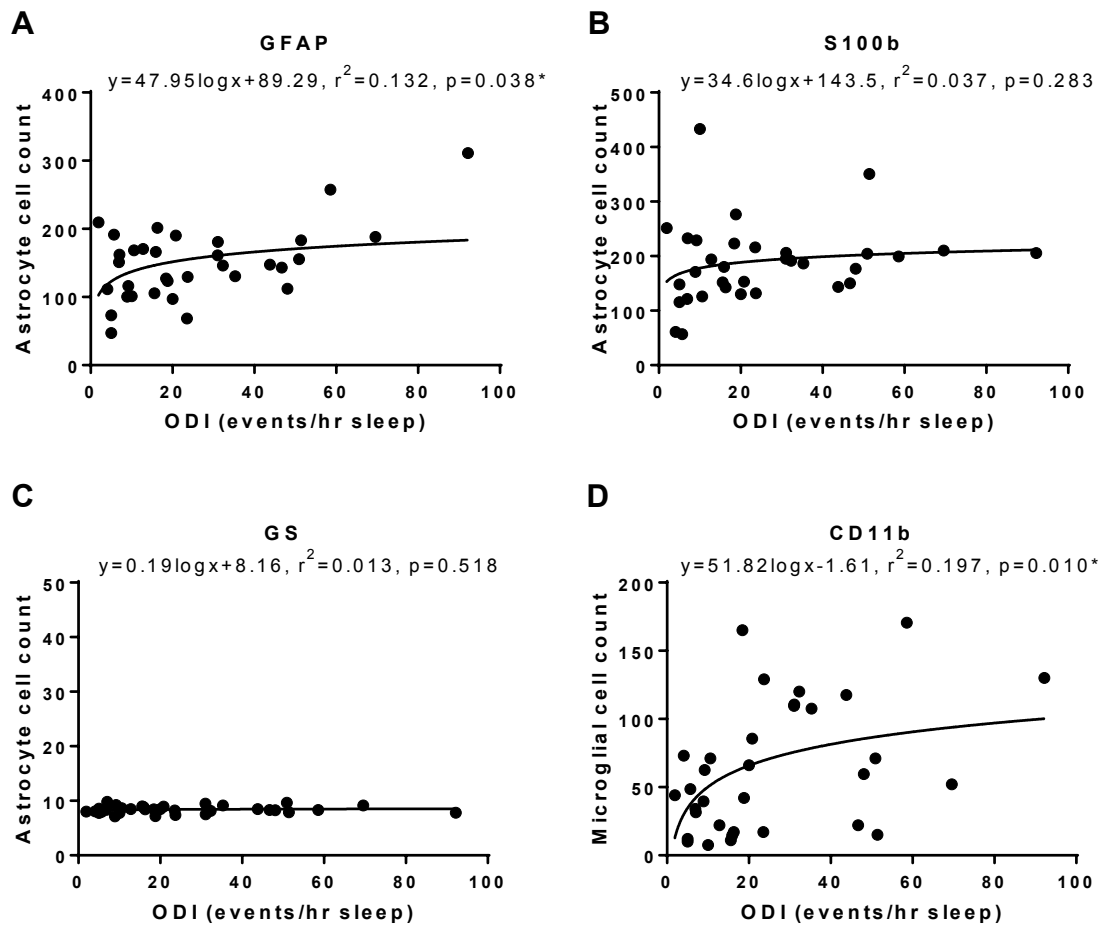


Figure 4.11 Glial cell counts in subiculum regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D). * indicates $p<0.05$.

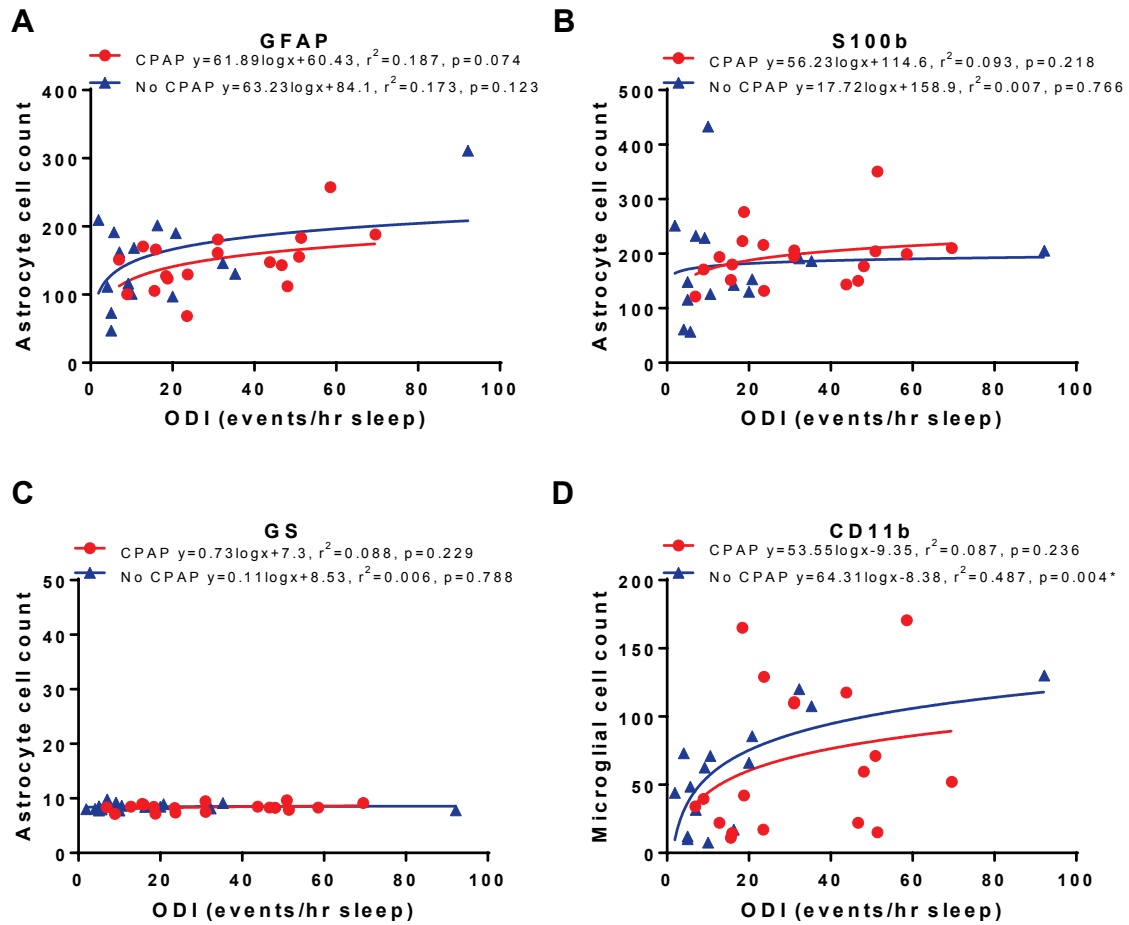


Figure 4.12 Glial cell counts in subiculum regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p<0.05$.

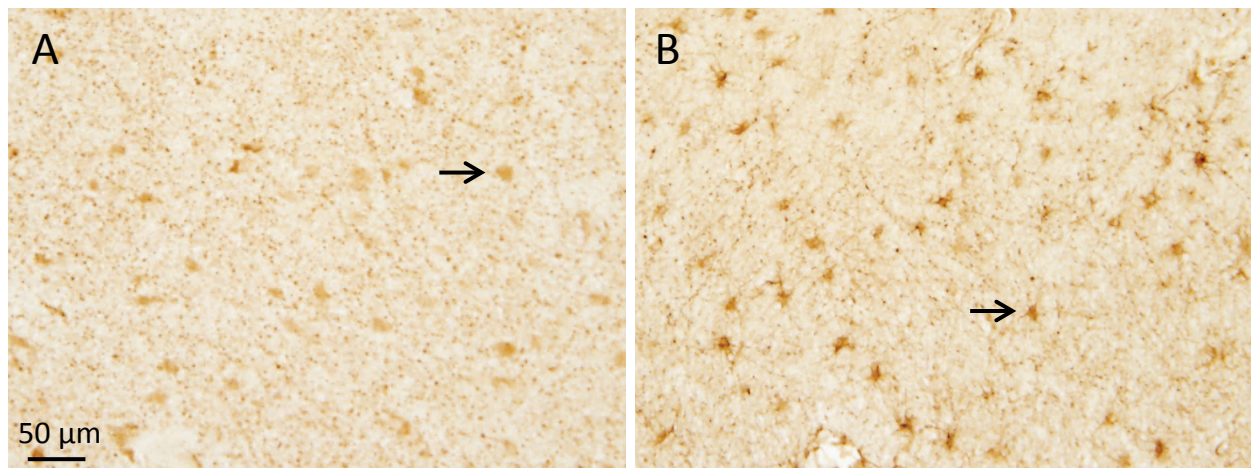


Figure 4.13 GFAP-positive astrocytes in the subiculum. Micrographs of GFAP-immunolabelled astrocytes (arrows) in the subiculum from a patient with low (A) and high (B) OSA, notice the increased number of labelled astrocytes in the right image compared to the left. Scale bar in A applies to both panels.

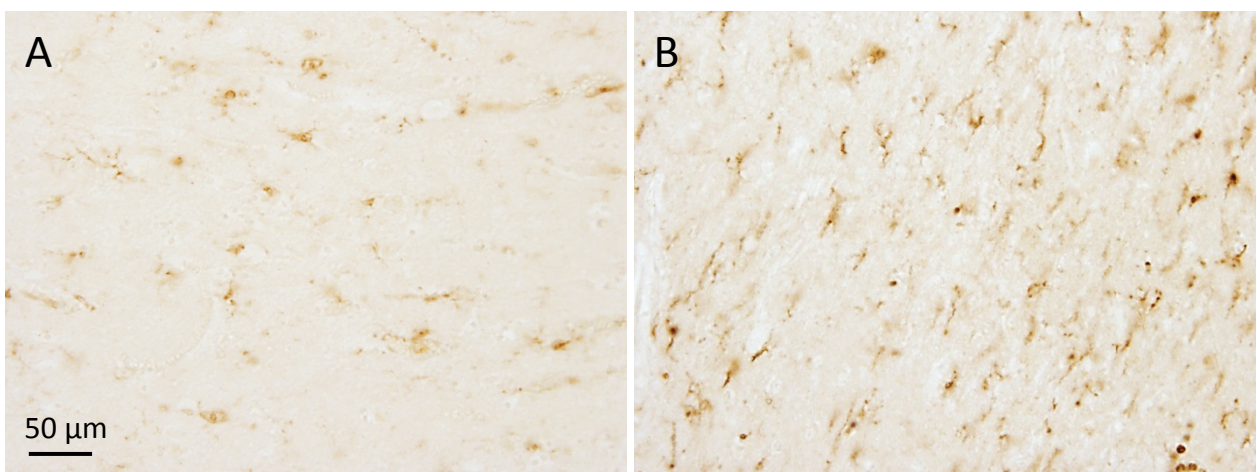


Figure 4.14 CD11b labelled microglia in subiculum. Micrographs of CD11b-positive microglia in the subiculum from a patient with low (A) and high (B) OSA, notice the increased number of labelled microglia in the right image compared to the left. Scale bar in A applies to both panels.

4.3.2.5 Astrocyte and microglia cell counts in the entorhinal cortex

In the entorhinal cortex, no relationships were found between ODI and any of the glial markers (Figure 4.15). When split by CPAP use, no significant relationships were seen (Figure 4.16).

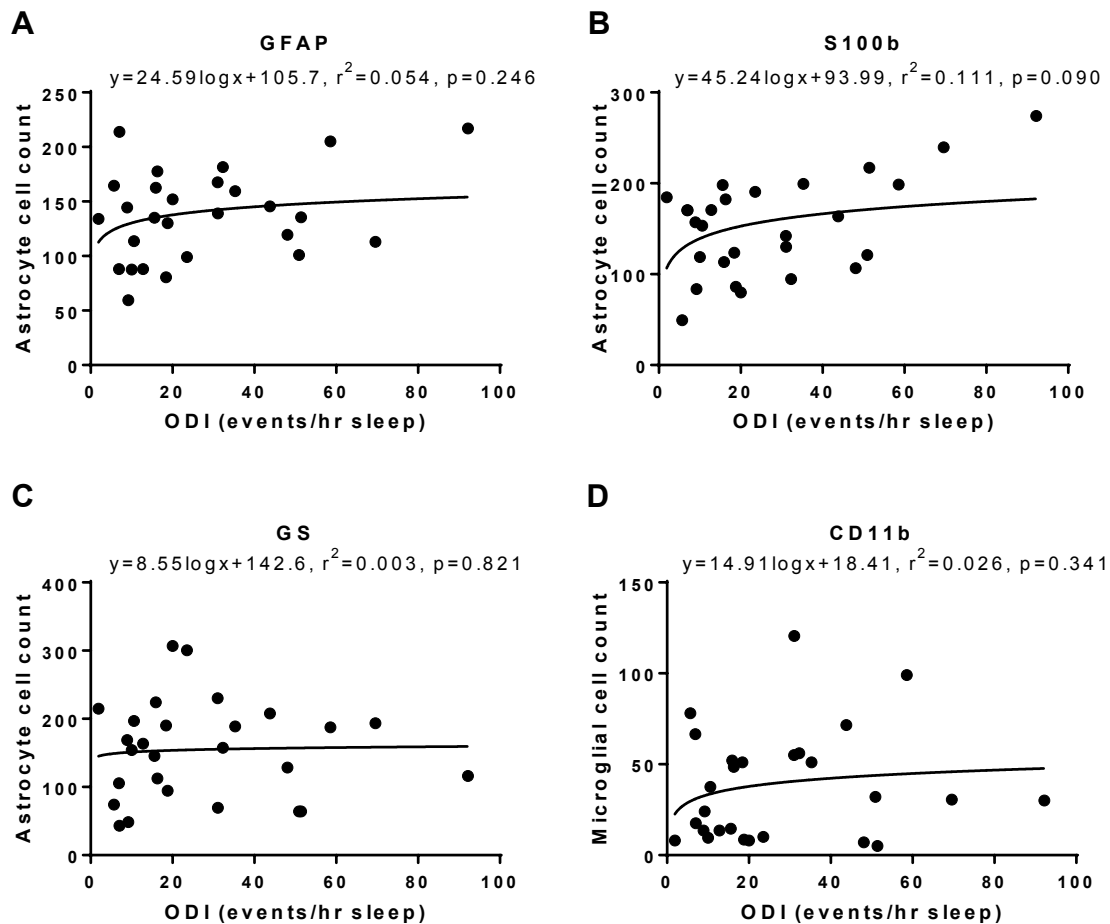


Figure 4.15 Glial cell counts in entorhinal cortex regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D). * indicates $p<0.05$.

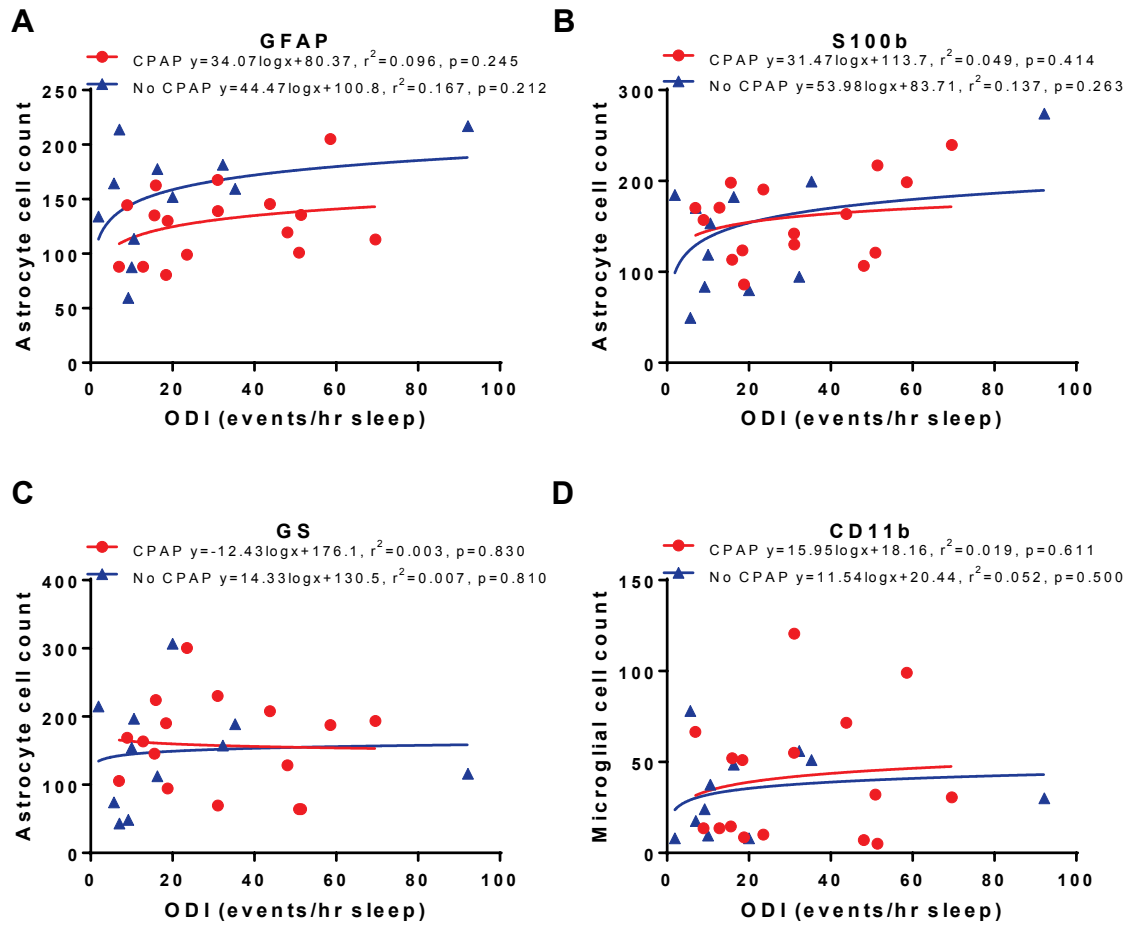


Figure 4.16 Glial cell counts in entorhinal cortex regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p < 0.05$.

4.3.3 Staining intensity of astrocytes and microglia

4.3.3.1 Astrocyte and microglia staining intensity in the hilus (CA4)

In the hilus, no relationships were found between OSA severity and the number of cells with increased staining intensity for any of the glial markers (Figure 4.17). When stratified by CPAP use no significant relationships were seen (Figure 4.18).

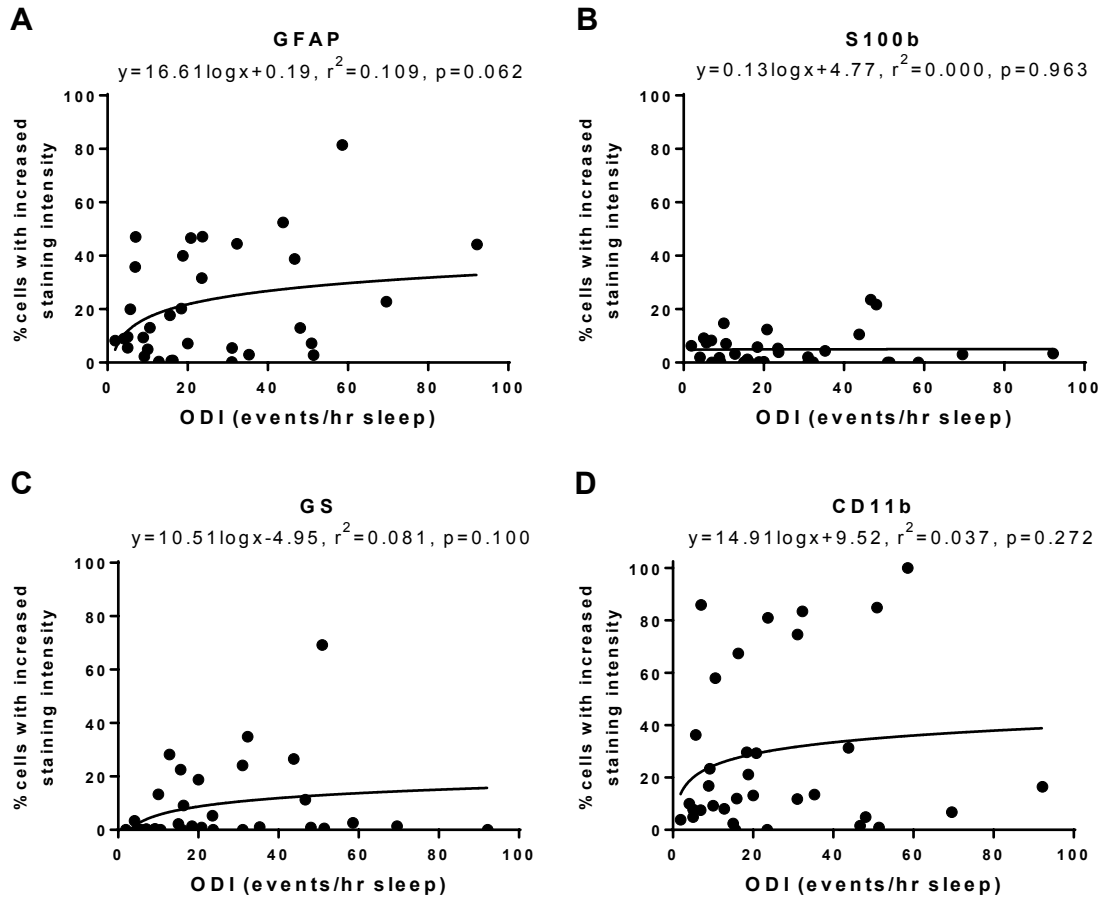


Figure 4.17 Glial staining intensity in the hilus regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D). * indicates $p < 0.05$.

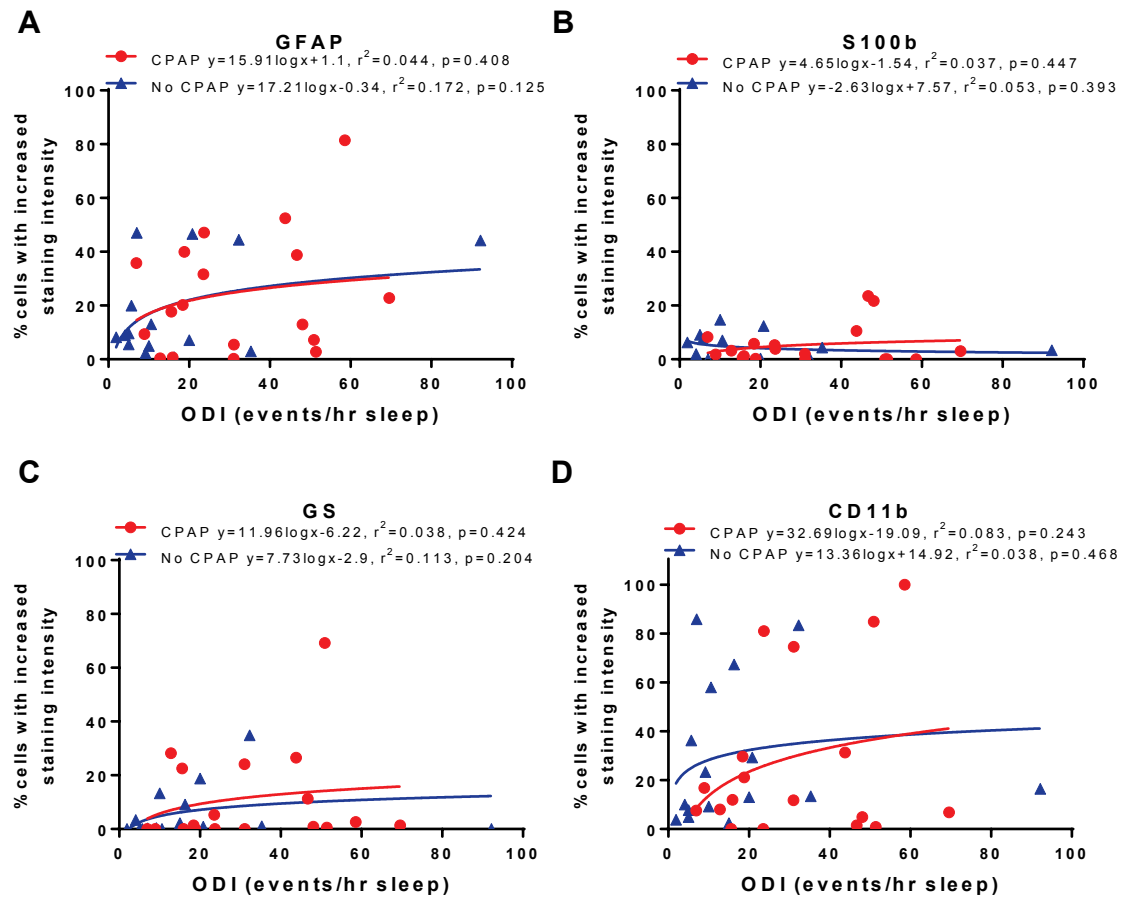


Figure 4.18 Glial staining intensity in the hilus regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p < 0.05$.

4.3.3.2 Astrocyte and microglia staining intensity in the CA3

In CA3 the number of cells with increased intensity of S100b immunostaining decreased with increasing ODI, $r^2=0.123$, $p=0.044$ (Figure 4.19 & 4.21). No significant relationships were found for the whole sample for the other glial markers. When stratified by CPAP use (Figure 4.20), CPAP non-users had a stronger but non-significant negative relationship between ODI and the percentage of cells with increased S100b staining, $r^2=0.227$, $p=0.062$, CPAP users also had a much stronger and non-significant relationship, $r^2=0.132$, $p=0.141$, however this relationship was in the opposite direction to the relationship for the whole sample. This indicates a loss of the relationship with CPAP use and a strengthening of the relationship with

no CPAP use. The percentage of cells with increased intensity of GFAP immunostaining was significantly related to ODI for CPAP non-users, $r^2=0.431$, $p=0.008$. Thus, the percentage of cells with more intense GFAP staining increased with increasing ODI for CPAP non-users but not for CPAP users. No significant relationships were found in the CA3 for the other glial markers.

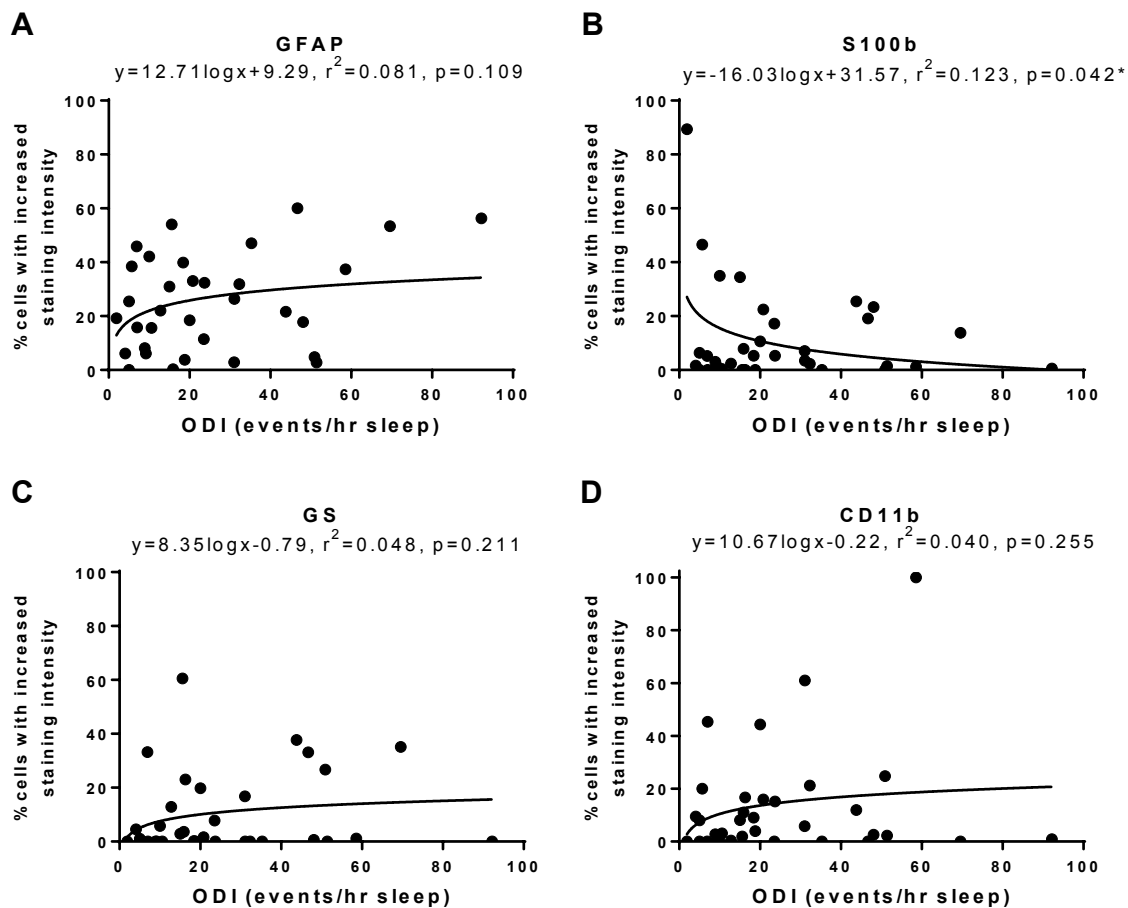


Figure 4.19 Glial staining intensity in CA3 regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D). * indicates $p < 0.05$.

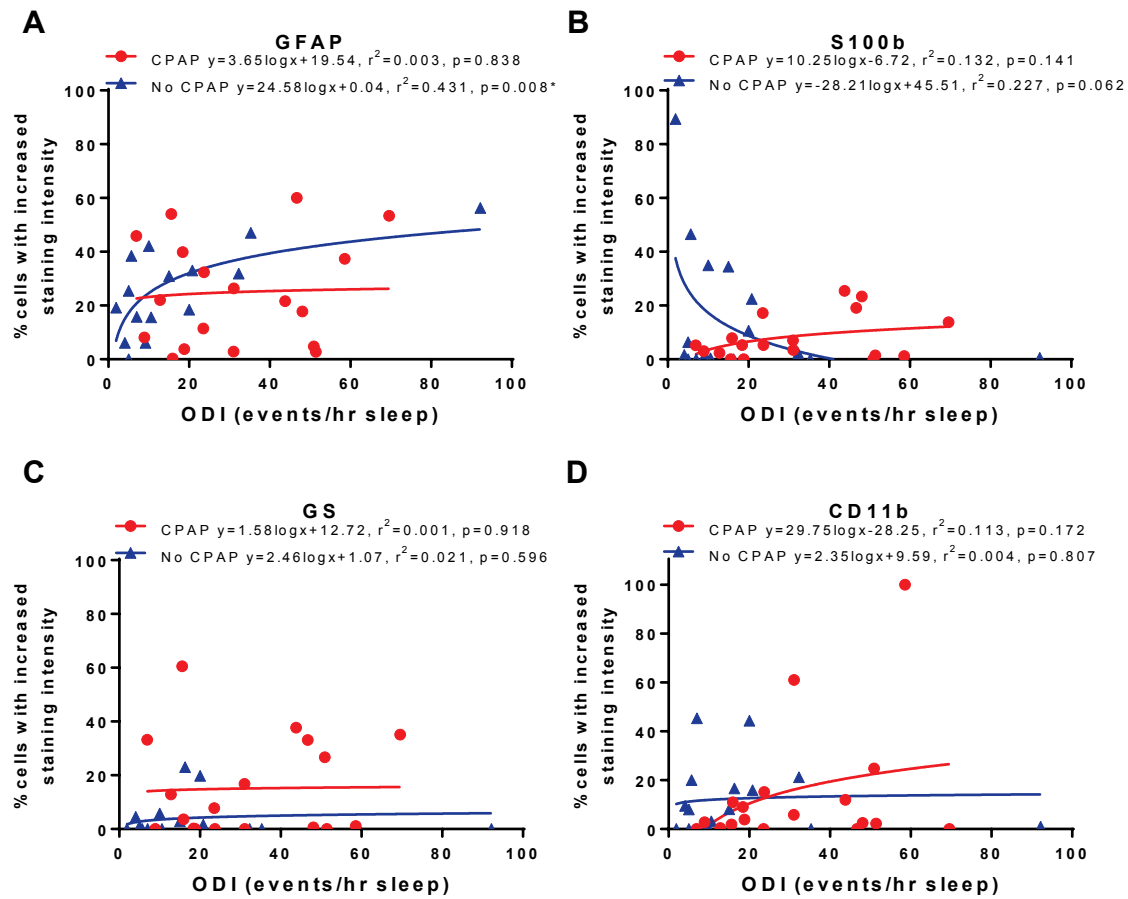


Figure 4.20 Glial staining intensity in CA3 regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D), separated by CPAP use. CPAP users are indicated by red circles and CPAP non-users are indicated by blue triangles. * indicates $p<0.05$.

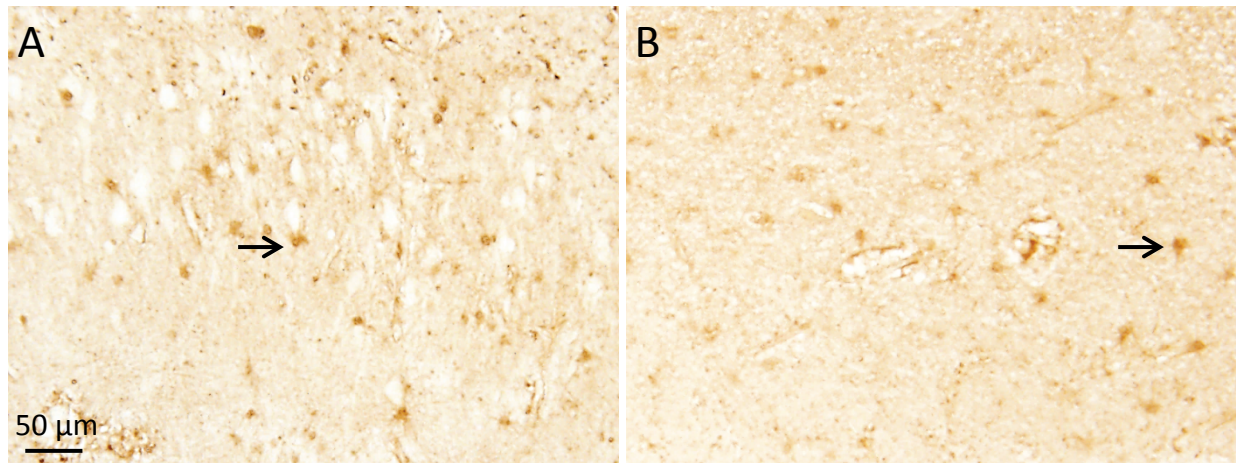


Figure 4.21 S100b-positive astrocytes in the CA3. Micrographs of S100b-immunolabelled astrocytes (arrows) in the CA3 from a patient with low (A) and high (B) OSA, notice the decreased number of intensely labelled astrocytes in the right image compared to the left. Scale bar in A applies to both panels.

4.3.3.3 Astrocyte and microglia staining intensity in the CA1

In the CA1 there were no significant relationships for any of the glial markers, with respect to the percentage of cells with increased staining intensity and ODI (Figure 4.22). When stratified by CPAP use, no significant relationships were seen (Figure 4.23).

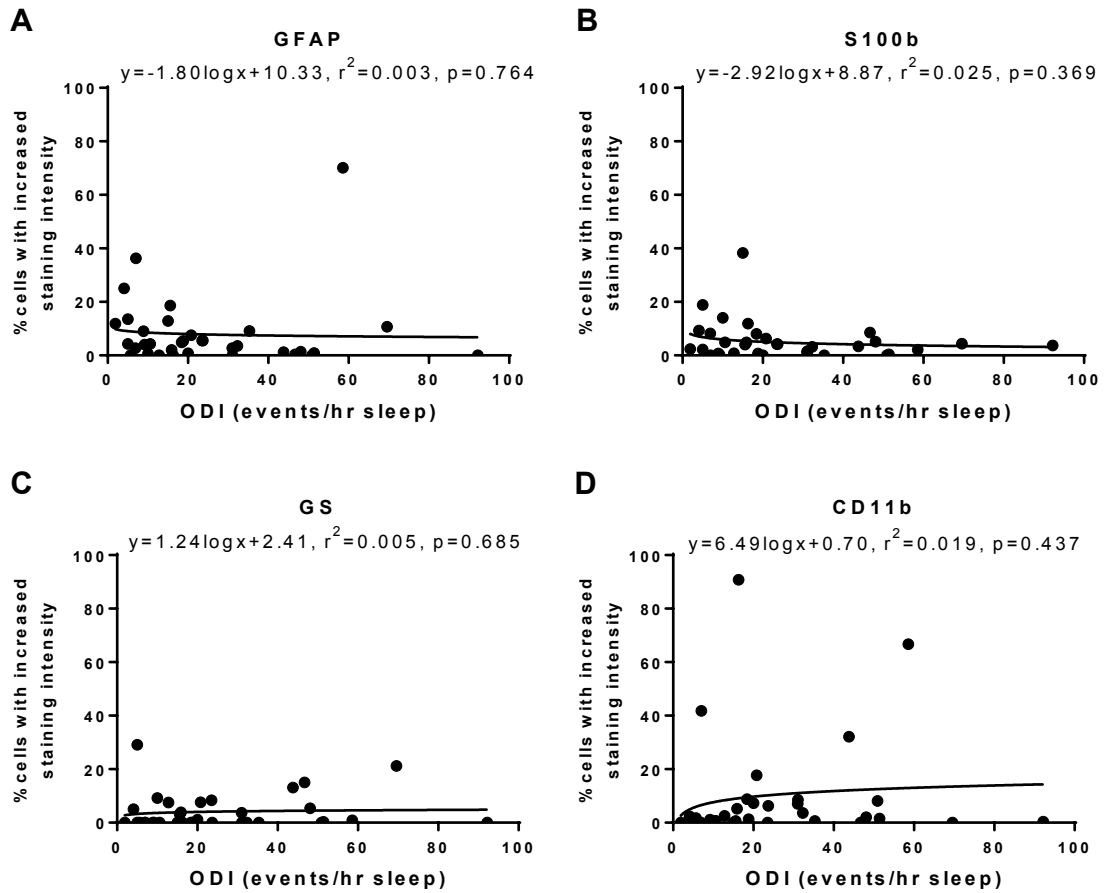


Figure 4.22 Glial staining intensity in CA1 regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D). * indicates $p < 0.05$.

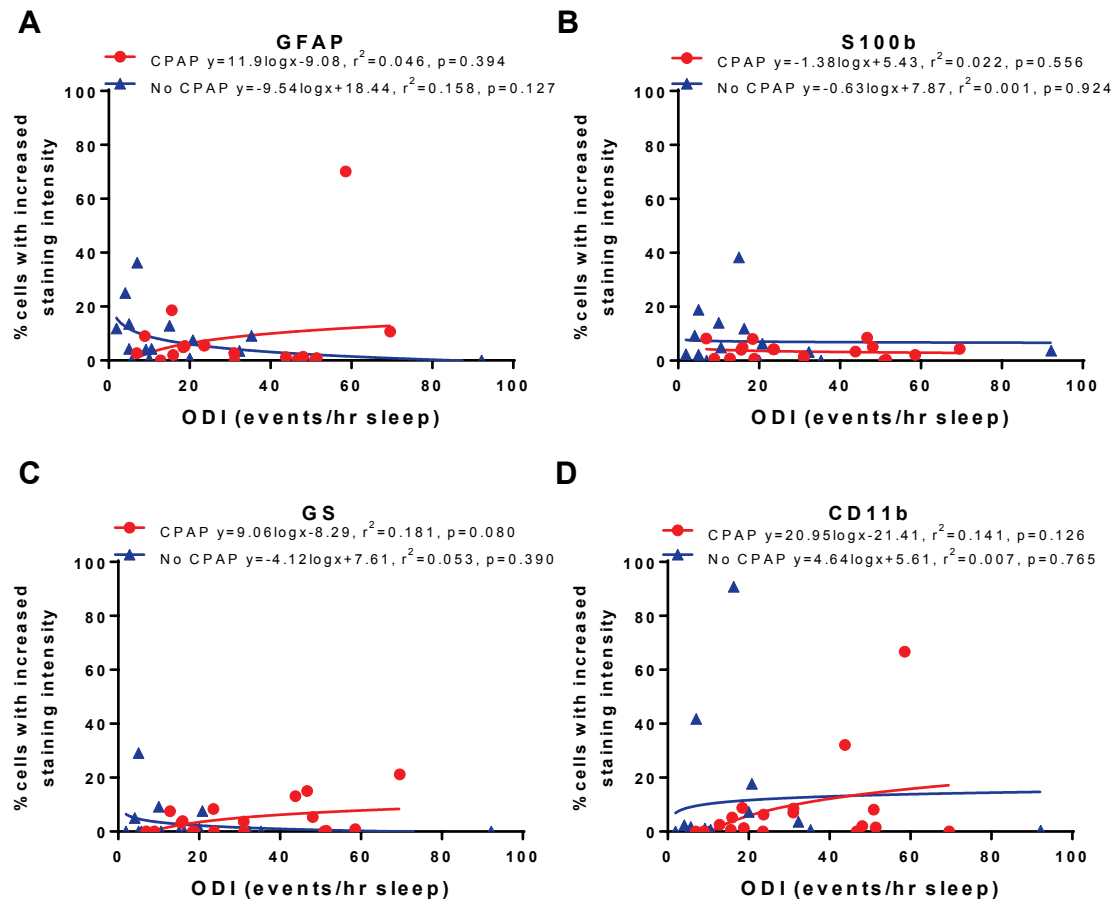


Figure 4.23 Glial staining intensity in CA1 regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p<0.05$.

4.3.3.4 Astrocyte and microglia staining intensity in the subiculum

In the subiculum, no relationships were found between ODI and the percentage of cells with increased immunostaining for any of the glia markers (Figure 4.24). No relationships emerged when the samples were stratified by CPAP use (Figure 4.25).

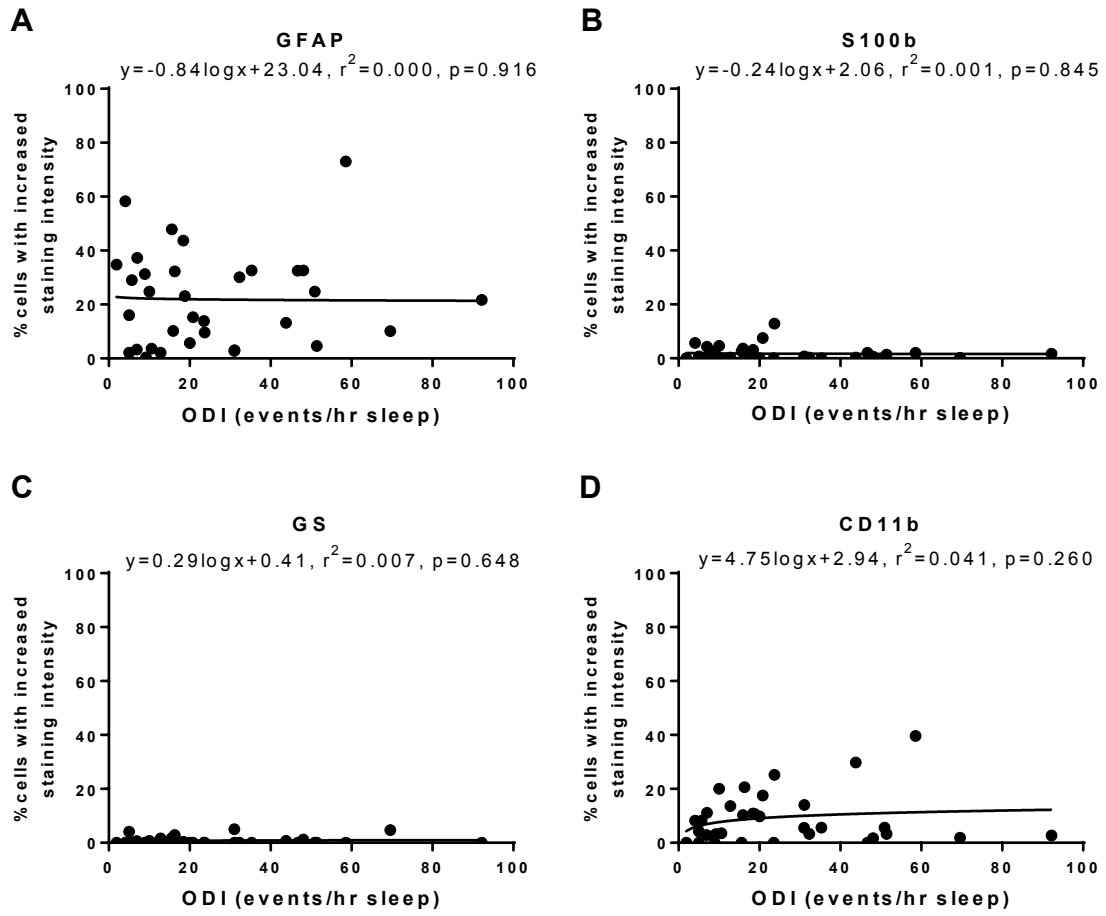


Figure 4.24 Glial staining intensity in subiculum regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D). * indicates $p < 0.05$.

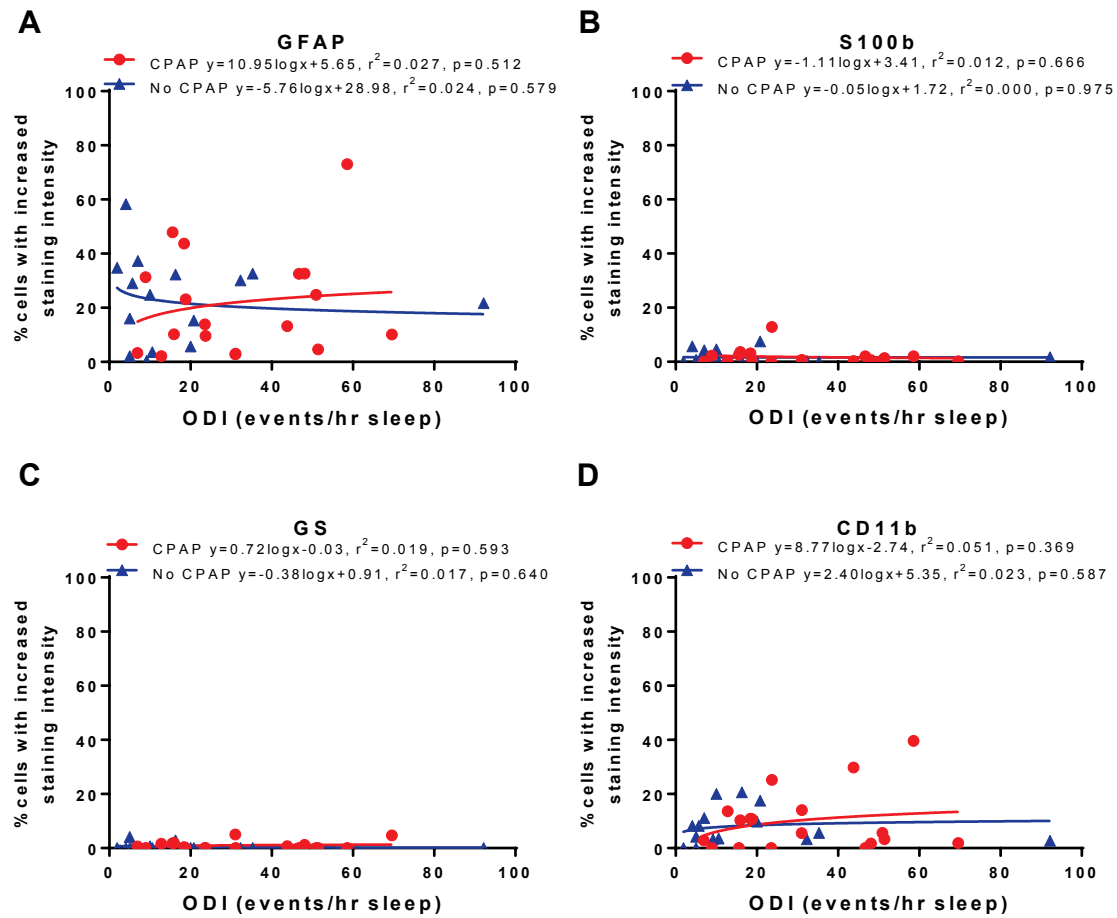


Figure 4.25 Glial staining intensity in subiculum regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p < 0.05$.

4.3.3.5 Astrocyte and microglia staining intensity in the entorhinal cortex

In the entorhinal cortex, no significant relationships were found between ODI and increased intensity of immunolabelling for S100b, GS or CD11b. However, the number of cells with increased intensity of GFAP immunostaining was significantly related to ODI, $r^2 = 0.178$, $p = 0.028$, (Figure 4.26 & 4.28). When stratified by CPAP use (Figure 4.27), CPAP users had a weak and non-significant relationship between ODI and the percentage of cells with increased GFAP staining, $r^2 = 0.075$, $p = 0.303$, whereas CPAP non-users had a much stronger and more significant relationship, $r^2 = 0.367$, $p = 0.048$.

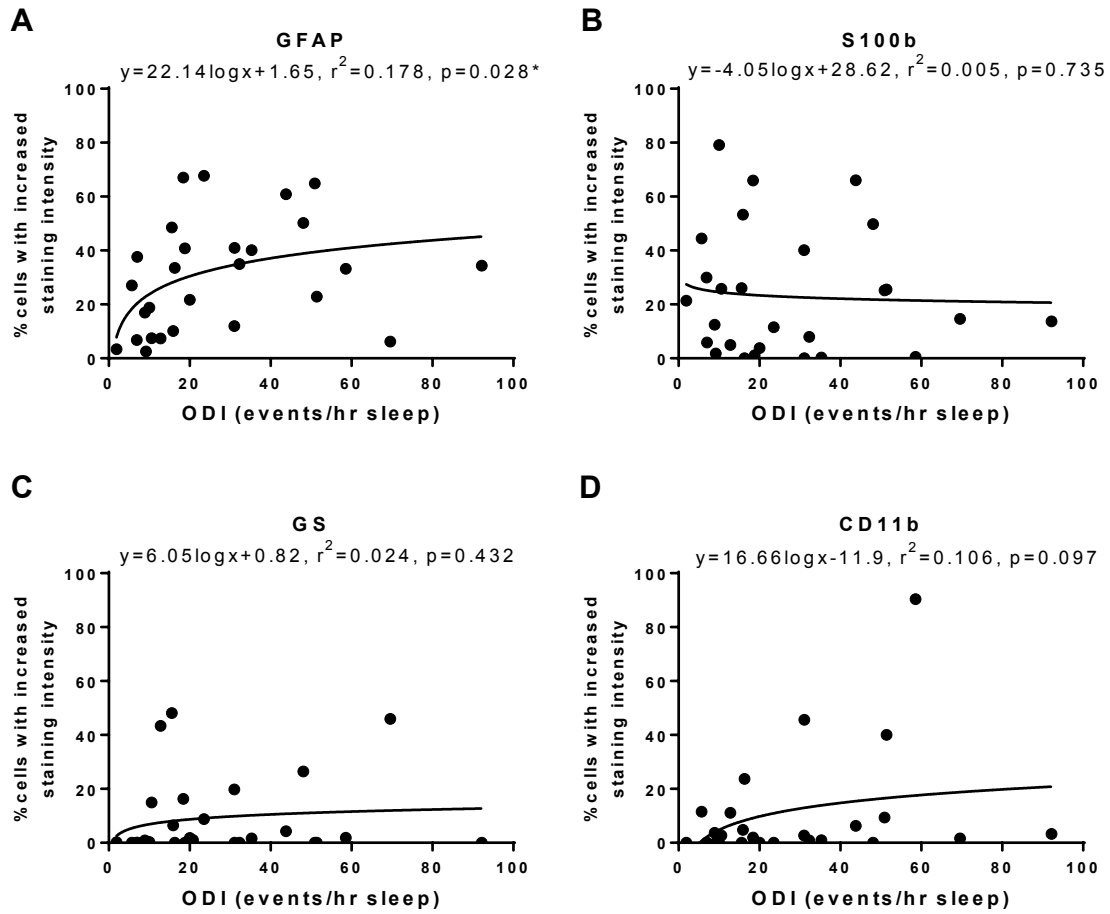


Figure 4.26 Glial staining intensity in entorhinal cortex regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D). * indicates $p < 0.05$.

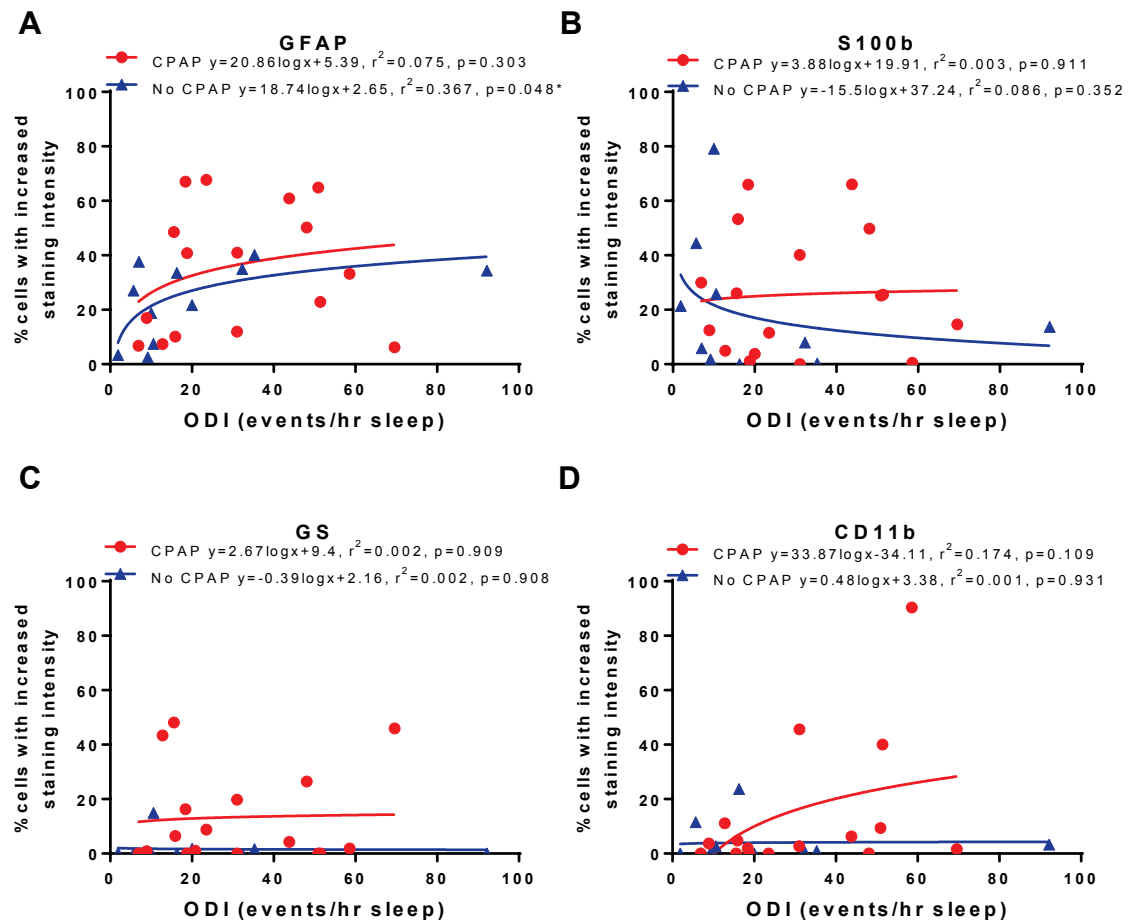


Figure 4.27 Glial staining intensity in entorhinal cortex regressed against OSA severity (ODI) for GFAP (A), S100b (B), GS (C) and CD11b (D), separated by CPAP. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p < 0.05$.

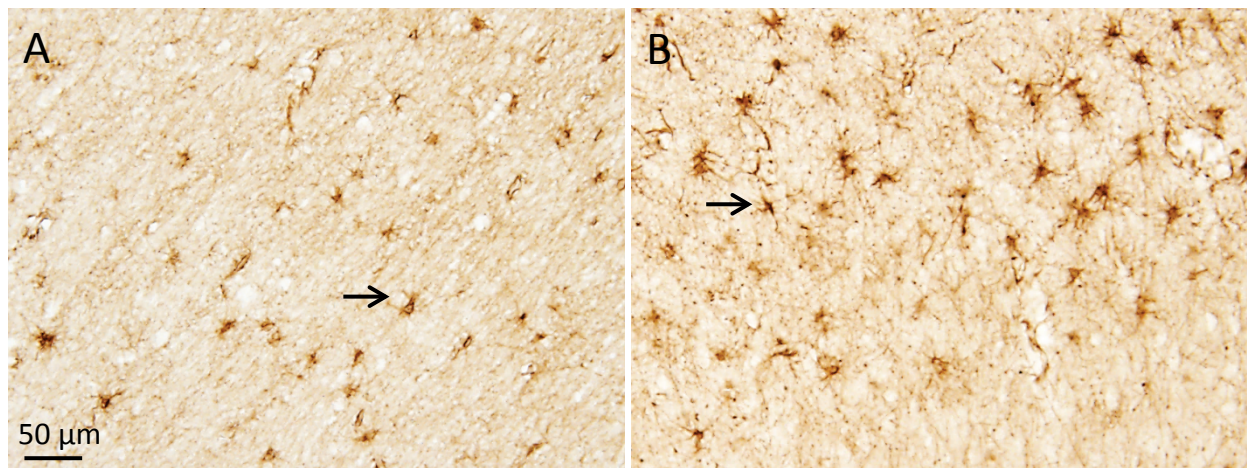


Figure 4.28 GFAP-positive astrocytes in the entorhinal cortex. Micrographs of GFAP-immunolabelled astrocytes (arrows) in the entorhinal from a patient with low (A) and high (B) OSA, notice the increased number of intensely labelled astrocytes in the right image compared to the left. Scale bar in A applies to both panels.

4.3.4 Microglia morphology

The morphology of microglial cells was assessed using a 5 step scale (Figure 4.29). The average morphology score for the low OSA and high OSA groups for each hippocampal region is illustrated in Figure 4.30. No significant differences were found between low- and high-OSA patients in any of the regions, indicating that microglial morphology was not related to OSA.

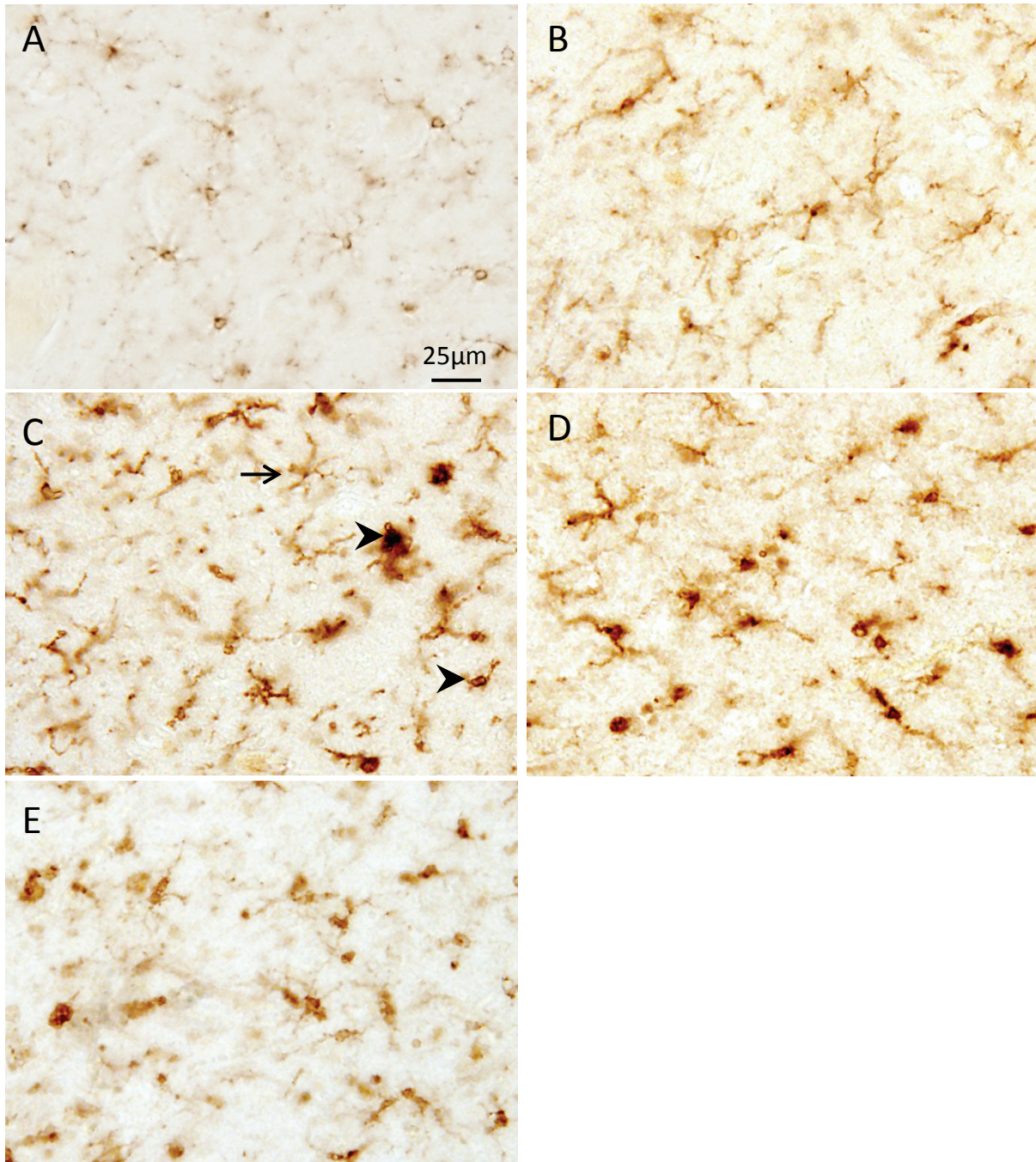


Figure 4.29 Microglia morphology classifications. Level 1, all resting microglia (A); level 2 mostly resting microglia (B); level 3, half resting (arrow), half activated (arrow head) (C); level 4, mostly activated (D); level 5, all activated (E). Scale bar in A is the same for all micrographs.

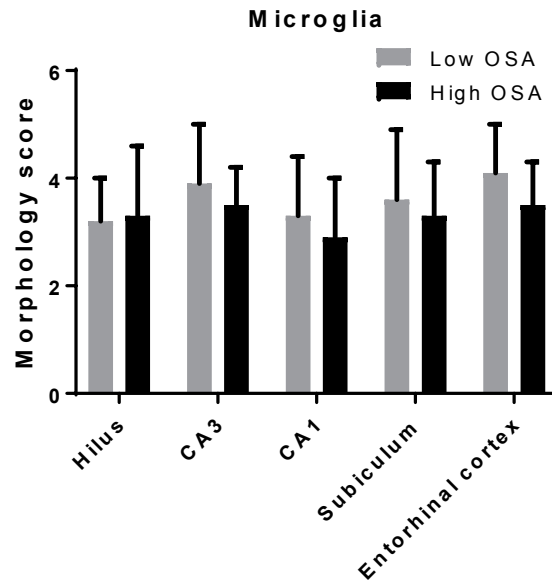


Figure 4.30 Microglial morphology score in low OSA (grey bars) and high OSA (black bars) for each of the 5 hippocampal regions.

4.3.5 Age and reactive gliosis

Where a significant relationship between ODI and cell count or staining intensity was found in the preceding sections, the reactive gliosis variable was then correlated with age to determine whether age influenced the relationship. Figure 4.31 shows the relationships between age and glial cell count. Microglial cell counts were significantly related to age in the hilus, $r^2=0.129$, $p=0.037$, and subiculum, $r^2=0.121$, $p=0.048$, and GFAP-positive cell counts in the subiculum were significantly related to age, $r^2=0.195$, $p=0.010$. However, in regards to the percentage of cells with increased staining intensity, no significant relationships were found, indicating that age did not influence the relationships between increased immunostaining intensity and ODI (Figure 4.32). Table 4.2 summaries the significant findings presented in the preceding sections.

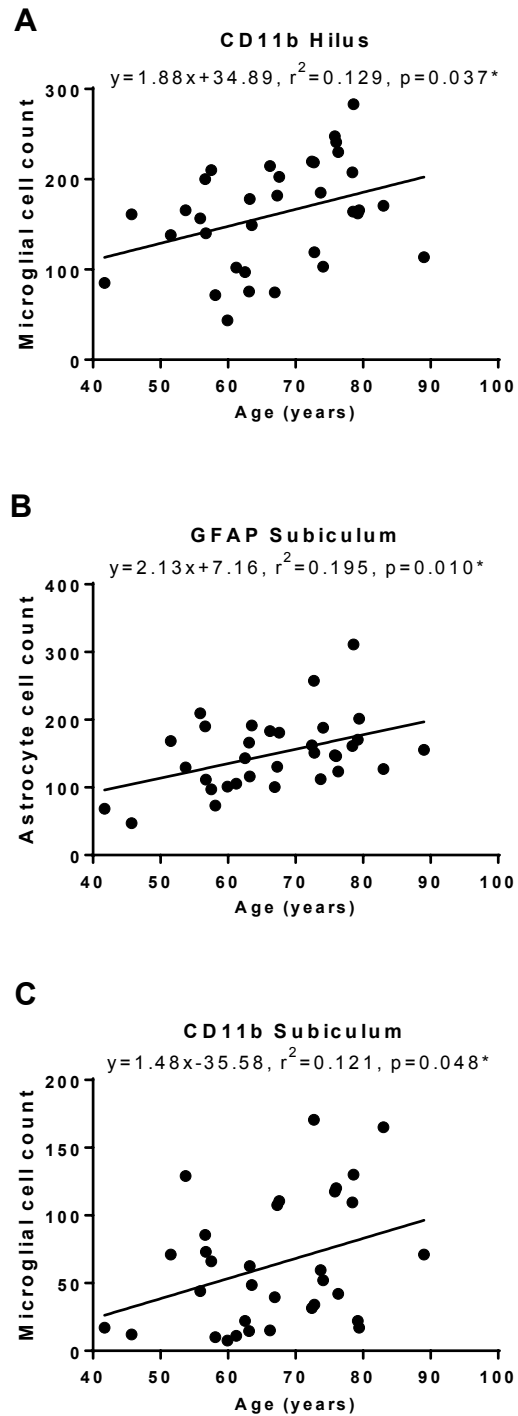


Figure 4.31 Patient age regressed against glial cell counts for CD11b at the hilus (A), GFAP (B) and CD11b (C) at the subiculum. * indicates $p < 0.05$.

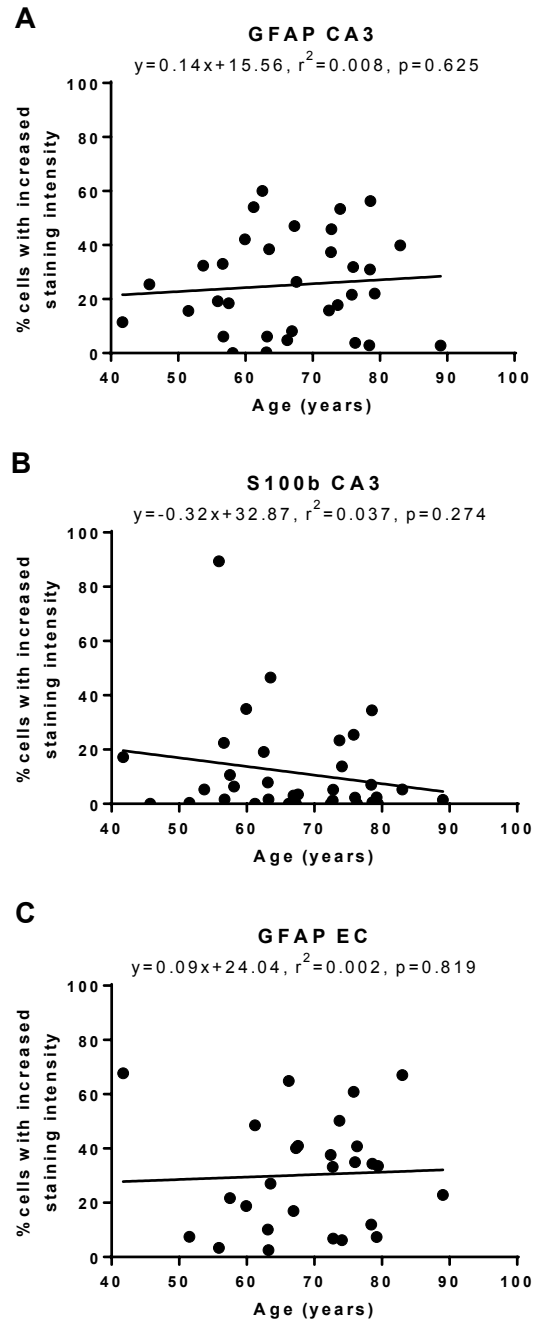


Figure 4.32 Patient age regressed against glial staining intensity for GFAP (A) and S100b (B) at the CA3 and GFAP at the entorhinal cortex (C). EC= entorhinal cortex * indicates $p<0.05$.

Table 4.2 Summary of the instances where a significant relationship was found between OSA severity and increased number of cells or the number of strongly-immunolabelled cells.

		Direction and magnitude of change as a function of increasing ODI	Effect of CPAP use (size of r^2)	Effect of not using CPAP (size of r^2)
Cell count	Hilus CD11b*	↑	↑	↑
Cell count	Subiculum GFAP*	↑	↑	↑
Cell count	Subiculum CD11b*	↑	↑	↑
Intensity	CA3 GFAP	none	↑	↑
Intensity	CA3 S100b	↓	↓	↓
Intensity	Entorhinal cortex GFAP	↑	↑	↑

↑ = smaller increase, ↑ = moderate increase, ↑ = larger increase, ↓ = moderate decrease, ↓ = larger decrease * = age may affect this relationship

4.4 Discussion

The present study found evidence of significant and regionally-specific reactive gliosis in the brains of OSA patients. While no glial reactivity was seen in the CA1, all other hippocampal regions examined had some gliosis, with the subiculum and CA3 being the most affected. Microgliosis, indicated by an increase in the number of microglial cells, was seen in the hilus and subiculum, and this increased with increasing ODI. Increased numbers of astrocytes were seen in the subiculum (GFAP), increased intensity of GFAP labelling was seen in the entorhinal cortex, and decreased intensity of S100b labelling was seen in the CA3, with all of these measures being correlated with increasing severity of OSA. Additionally, all of these relationships were stronger for CPAP non-users and most were diminished in CPAP users, indicating a mostly protective effect of CPAP treatment for reactive gliosis. Patient age was found to be related to the number of microglial cells in the hilus and subiculum and the number of GFAP-positive astrocytes in the subiculum. Reactive gliosis is known to increase in the brain with age (Cotrina & Nedergaard, 2002), providing support for the findings here. Due to the small sample size of the present study, the individual effects of age and OSA

could not be determined. However, none of relationships with intensity of labelling were related to age suggesting that OSA is associated with reactive gliosis independent of age.

Neuroinflammation has been suggested as a contributing factor to the structural changes commonly found in OSA patients. Decreased regional brain volume and reduced white matter integrity have been found in the hippocampus and surrounding regions in OSA patients compared to controls (Canessa et al., 2011; Kumar et al., 2012; Macey et al., 2002), and are indicative of tissue injury. Any brain tissue injury typically induces neuroinflammation, including reactive gliosis. MRS studies of the brains of OSA patients have also found metabolite changes thought to be indicative of neuronal injury and reactive gliosis (O'Donoghue et al., 2012; Sarchielli et al., 2008; Sharma et al., 2010). Authors of such studies often suggest that changes to glial cells and inflammatory processes may be an underlying mechanism, however all of these studies have used indirect methods to infer the presence of neuroinflammation in OSA. The present study provides the first direct evidence of neuroinflammation in response to increasing OSA severity. Significant reactive gliosis was detected in relation to increasing ODI despite the existence of possible confounding factors, such as the *post-mortem* interval and the cause of death which may have induced reactive changes in glial cells. The most frequent changes observed in the present study were for microglia and GFAP-positive astrocytes.

Increased numbers of GFAP-positive astrocytes were found in the subiculum and an increased percentage of cells with high GFAP immunoreactivity were found in the entorhinal cortex, as well as in the CA3 of non-CPAP users. The increased GFAP expression may explain why CA3 is considered to be more resistant to hypoxic damage than CA1, which showed no change in the number or intensity of GFAP-labelled astrocytes. GFAP may aid in improving blood flow in hypoxic conditions, since GFAP-null mice have been shown to have poorer cerebral blood flow after a transient ischaemic episode compared to wild-type mice (Brenner, 2014). Thus the upregulation of GFAP after IH or OSA may be associated with an increase in blood flow in those regions.

Intensity of S100b labelling decreased in the CA3 region with increasing OSA severity. S100b is known to be secreted by astrocytes in response to injury (Griffin et al., 1989; Matsui et al., 2002), suggesting that injury would result in a decrease in intracellular S100b and an increase in extracellular S100b. This is supported by the present finding as only intracellular S100b was measured. Extracellular S100b release stimulates proinflammatory cytokine

release from astrocytes, promoting further reactive gliosis (Donato et al., 2009; Matsui et al., 2002). The CA3 was the only region where S100b labelling decreased in the present study; interestingly it was also the only region where additional astrocyte activation was seen (increased GFAP labelling in non-CPAP users). While the release of S100b in low concentrations is neuroprotective, high concentrations are neurotoxic (Donato et al., 2009). OSA patients have increased serum S100b levels (Braga et al., 2006; Duru et al., 2012), suggesting S100b is being released by astrocytes in the brain to prevent neurotoxicity. The present finding of decreased intracellular S100b in astrocytes supports this.

Microglial cells were increased in numbers in the hilus and subiculum, indicating neuroinflammation in these regions, however no change in the intensity of CD11b labelling was found. This indicates that microglia were migrating to or proliferating in the hilus and subiculum, but were not increasing their production of CD11b or Mac-1. An array of inflammatory markers is induced in response to microglial activation, and while Mac-1 (CD11b) is upregulated in neurodegeneration (Block et al., 2007), it does not seem to be upregulated in OSA. Indeed, an animal study of IH found microglial proliferation and upregulation of some inflammatory markers, while other inflammatory markers remained unchanged (Sapin et al., 2015). The present study found no change in CD11b expression, however there may be differences in the upregulation of other inflammatory markers. Perhaps future studies could investigate other microglial markers to better characterise the neuroinflammatory response in this cell type.

The morphology of microglial cells varied across brains and regions with many activated microglia present, however activated/resting morphology was not related to ODI. This may be due to the *post-mortem* interval which was unknown in the present study. It has been recently been found in mice that microglia had an activated and more amoeboid morphology after a longer *post-mortem* interval, whereas microglia had a rested, stellate morphology when there was no *post-mortem* delay (Gonzalez-Riano et al., 2017). While this study was performed in mice and different fixation methods were used for the different *post-mortem* delay conditions, it does suggest that the morphology of microglia could be influenced by *post-mortem* delay.

Reactive gliosis is a continuum of glial changes rather than an all or nothing process (Pekny, Wilhelmsson, & Pekna, 2014). In response to a localised injury such as a stroke, the process of reactive gliosis is well defined (Gao et al., 2013; Sofroniew, 2009). Microglia mount an

inflammatory response and migrate to the injury site which then activate astrocytes that upregulate GFAP and other proteins in order to form a glial scar that ultimately protects the rest of the brain from the injury (D. Zhang et al., 2010). This is a protective mechanism that isolates the injured tissue to prevent the spread of injury (Sofroniew, 2009). However, the pattern of gliosis observed in the present study did not match a sequence of initial microglial activation followed by astrocyte activation. One region with increased numbers of microglia had no astrocyte activation (hilus), the other region with increased numbers of microglia (subiculum) had astrocyte activation (GFAP). Indeed, the CA3, and entorhinal cortex displayed astrocyte activation in the absence of increased numbers of microglia, which might indicate that astrocytes can be activated independently of microglia. Indeed, astrocyte activation prior to and in the absence of microglial activation has been previously found in mice exposed to hypoxia (Tadmouri, Champagnat, & Morin-Surun, 2014).

The heterogeneous response of astrocytes to injury has been well documented (Anderson et al., 2014; Zamanian et al., 2012). Two types of variability were observed in the present study: i) variability among markers within a given region; and ii) variability between regions for a given marker. Marker variability within a given region of the hippocampus will be discussed first. In the subiculum for example, the number of microglial cells increased and the number of GFAP-positive astrocytes increased, but no changes were seen for S100b or GS. Indeed, subpopulations of astrocytes exist that differentially express various astrocytic markers and have different responses to injury. While co-localisation of GFAP and S100b in astrocytes is common in reactive gliosis (Wagner et al., 2013), large differences between the estimated density of GFAP-positive and S100b-positive astrocytes have been found throughout the brain (Emsley & Macklis, 2006). Specifically in the mouse hippocampus, Emsley and colleagues found that there were approximately five times as many GFAP-positive astrocytes than S100b-positive astrocytes. Further, there is a subpopulation of astrocytes that do not express GFAP but do express other astrocyte markers including S100b and GS (Robinson, 2000; Walz, 2000). The present findings of an increased number of GFAP-positive astrocytes only in the subiculum, increased intensity of GFAP labelling in the entorhinal cortex and CA3 (CPAP non-users only), decreased intensity of S100b labelling in the CA3 and no change in GS expression, support the idea of astrocyte subpopulations that have different injury responses.

Variability was also seen across different hippocampal regions, with evidence of reactive gliosis in some areas and not others, suggesting regional sensitivity to hypoxia. Indeed,

different regions of the hippocampus are differentially vulnerable to hypoxia: CA1 is more vulnerable, and CA2 and CA3 are more resistant to hypoxic injury (Duvernoy et al., 2013). Further, regional variability has been shown in a study investigating the microglial density in the hippocampus after chronic (6 and 24 weeks) IH exposure (Sapin et al., 2015). Increased microglial density occurred in the dorsal hippocampus but not in the ventral hippocampus.

Since the CA1 is the region of the hippocampus that is most sensitive to hypoxia, the present study expected to detect significant gliosis in this region, however the results did not support this expectation. The phenomenon of adaptation or ‘preconditioning’ may explain this surprising result. Preconditioning refers to a situation where cells are exposed to a sublethal dose of an aversive stimulus several days prior to exposure to a lethal dose. Typically, the sublethal dose induces cells to upregulate their metabolic defences, so that when a previously lethal dose is subsequently administered, the cells have adequate defences to survive. Cells that are repeatedly exposed to aversive conditions are eventually able to adapt to the changed conditions and no longer exhibit a stress response. Hypoxia is one stressor where brain cells have been shown to benefit from preconditioning (Do Val-da Silva et al., 2016). For instance, decreased reactive gliosis was found in neonatal animals exposed to hypoxic preconditioning prior to an ischaemic injury, when compared to neonates that did not receive the preconditioning (C. Y. Chen et al., 2015). These results were found for both microgliosis (CD11b-positive cells) and astrogliosis (GFAP-positive cells). It is notable that Lavie and Lavie (2009) have postulated that OSA may precondition individuals to intermittent hypoxia and thus improve their likelihood of survival if that individual experiences a transient ischaemic attack. When interpreted within this perspective, the present results may indicate that the CA1 in OSA patients is so well adapted to hypoxia that it no longer stimulates gliosis. It would be interesting to test this idea in an animal model of chronic IH.

The present study found that CPAP use significantly reduces the extent of gliosis, and hence is likely to also reduce the extent of neuronal injury that triggers gliosis. All of the significant glial relationships with ODI showed a stronger relationship for CPAP non-users than CPAP users, although some did not reach statistical significance due to the small sample size. This finding supports previous studies that found a reduction in proinflammatory cytokines in the serum of OSA patients after CPAP treatment indicating decreased inflammation (Akinnusi, Jaoude, Kufel, & El-Solh, 2013; S. Q. Wu et al., 2016; Xie, Pan, Ren, Du, & Guo, 2013). Further, this reversibility of neuroinflammation may be the basis of some of the cognitive improvements found after CPAP treatment. In particular, memory impairments have been

found to recover after three months of CPAP treatment (Canessa et al., 2011); the results of the present study suggest that this may be due to the reversibility of neuroinflammation in the hippocampus. However, not all studies have reported cognitive improvements with CPAP treatment (O'Donoghue et al., 2012), suggesting that some of the injury caused to the brain by OSA is irreversible. The effective treatment of neuroinflammation with CPAP in the present study may have implications for the treatment of other conditions that involve neuroinflammation, including AD.

Late stage AD is associated with extensive reactive gliosis that is characterised by hypertrophic astrocytes, an upregulation of GFAP and S100b expression, a decrease in GS and increased reactive microglia throughout the hippocampus (Griffin et al., 1989; Mrak, 2012; Robinson, 2000; Verkhratsky et al., 2010). This situation contrasts with the results of the present study that found evidence of reactive gliosis in different regions of the hippocampus with different markers. Inflammatory markers increase in the vicinity of NFTs and A β plaques in AD (Dickson et al., 1988; Sheng et al., 1997). The present study did find increased intensity of GFAP staining in the entorhinal cortex, the first region to show NFTs and A β deposition, however no microglial activation was found in this region. Another characteristic feature of AD is the presence of hypertrophic, GFAP-positive astrocytes that surround A β fibrillary plaques (Nagele et al., 2003; Robinson, 2001). The present thesis (Chapter 3) detected moderate burdens of A β plaques in the hippocampus of high OSA patients, yet was unable to detect any examples of hypertrophic, GFAP-positive astrocytes surrounding these A β plaques. Furthermore, the pattern of reactive gliosis observed in OSA brains seems to be unrelated to the pattern of NFT accumulation (Chapter 3). NFTs were rarely found in the subiculum, and were only observed in the CA3 in the later NFT stages (Stage 3 & 4). By contrast, the present study found that the subiculum and CA3 are the regions most affected by reactive gliosis. Taken together, these findings suggest that glial cells in OSA are activated in response to intermittent hypoxia, rather than by the presence of A β plaques and NFTs.

The present study has shown that the extent of neuroinflammation in the hippocampus of OSA patients increases with increasing disease severity. The spatial patterns of astrogliosis and microgliosis observed were dissimilar to the patterns usually seen in AD. Further, most of the neuroinflammatory changes were successfully attenuated with CPAP treatment suggesting that neuroinflammation in OSA is reversible. This finding may provide a basis for understanding the cognitive improvements that occur with CPAP treatment.

Chapter 5 Neuropathological investigation of cell layer thickness and myelination in the hippocampus of obstructive sleep apnoea patients.

Obstructive sleep apnoea (OSA) is commonly associated with memory impairments. Memory processing occurs in the hippocampus and there are specific neural circuits in the hippocampus that process different types of memory. MRI studies have found volumetric loss in the hippocampus of OSA patients compared to controls. However, MRI does not have the spatial resolution to detect atrophy in the specific regions of the hippocampus that are related to memory circuits. The present study performed histopathological investigations on autopsy brain tissue from OSA patients to examine whether the cortical thickness and myelination of the hippocampus and entorhinal cortex vary as a function of OSA severity. OSA severity was related to cortical thinning in the molecular layer of the dentate gyrus ($r^2=0.136$, $p=0.038$), the CA1 (overall, $r^2=0.135$, $p=0.039$; layer 1, $r^2=0.157$, $p=0.025$; layer 2, $r^2=0.255$, $p=0.003$; and layer 3, $r^2=0.185$, $p=0.014$) and some layers of the entorhinal cortex (layer 1, $r^2=0.186$, $p=0.028$; trend in layer 3, $r^2=0.124$, $p=0.078$). OSA severity was also related to decreased myelin staining in the deep layers but not the superficial layers of the entorhinal cortex (layer 6, $r^2=0.282$, $p=0.006$; deep white matter, $r^2=0.390$, $p=0.001$). CPAP use was related to less severe reductions in cortical thickness, suggesting a protective effect. The same effect was not observed for demyelination. The regions of decreased cortical thickness and less myelin staining overlap substantially with key parts of the hippocampal circuitry associated with memory. This correspondence has implications for the memory impairments seen in OSA patients and the beneficial effect of CPAP treatment.

5.1 Introduction

Obstructive sleep apnoea (OSA) is the intermittent cessation of breathing throughout sleep, leading to frequent episodes of hypoxia, severe sleep disruption, and consequent deficits in memory, attention and executive functioning (Canessa et al., 2011; Ferini-Strambi et al., 2003). Memory processing occurs in the hippocampus, a structure in the inferior part of the temporal lobe of the brain. Neuroimaging research has found gross volumetric changes in the brains of OSA patients (Canessa et al., 2011). Specifically, there is reduced hippocampal volume in OSA patients when compared to controls (Macey et al., 2002; Weng et al., 2014), which is correlated with deficits in verbal, visuospatial and working memory (Canessa et al., 2011; Gale & Hopkins, 2004).

It has been suggested that OSA patients experience regional loss of hippocampal grey matter, but such loss has not been demonstrated directly. MRI studies have reported that OSA patients have an increased hippocampal sulcus width, and the extent of this width is correlated with OSA severity (Akhan, Songu, Ayik, Altay, & Kalemci, 2015). An increased sulcus width could indicate reduced volume of the grey matter on either side of the hippocampal sulcus, which includes the dentate gyrus and CA1/subiculum. In this context, it is interesting that CPAP treatment of OSA patients increases the volume of their CA1 and dentate gyrus (H. Kim et al., 2016). However, MRI studies lack the spatial resolution to be able to resolve differences in the thickness of individual cortical cell layers. A capacity to do this would be very useful, since specific layers are associated with particular aspects of the memory circuitry, and hence reductions in the thickness of specific layers might provide insights into the circuits that are most affected in OSA.

The precise neural circuitry involved in memory formation, storage and retrieval is complex and not entirely understood. However, two functional neural circuits have been identified: the polysynaptic (trisynaptic) and direct (monosynaptic) pathways. The *polysynaptic pathway* is thought to process memories that involve spatial reference, as well as episodic memories (memory of personal experiences and events). The polysynaptic pathway may be important for the acquisition of new memories (Llorens-Martín et al., 2014). This pathway begins in the entorhinal cortex, specifically in layer 2 neurons of the six-layered cortex. These neurons send axons to the molecular layer of the dentate gyrus via the so-called ‘perforant pathway’. In turn, neurons in the dentate gyrus project to the CA3 region via specialised axons called ‘mossy fibres’. Pyramidal neurons in the CA3 region then project to CA2 and CA1 via branched axons called ‘Schaffer collaterals’. Pyramidal neurons in CA1 send axons through the subiculum to terminate in layer 5 of the entorhinal cortex, completing the loop (Figure 5.1) (Duvernoy et al., 2013; Llorens-Martín et al., 2014).

The circuitry of the *direct hippocampal pathway* is more straightforward. It originates in neurons located in layer 3 of the entorhinal cortex, which project to and synapse in the CA1 region. Pyramidal neurons in CA1 then send efferents back to the deep layers of entorhinal cortex, with some travelling via the subiculum (Figure 5.1) (Duvernoy et al., 2013). Semantic memory (memory of facts and general knowledge) is processed via this pathway, and it may also be involved in maintaining the stability of older memories (Llorens-Martín et al., 2014).

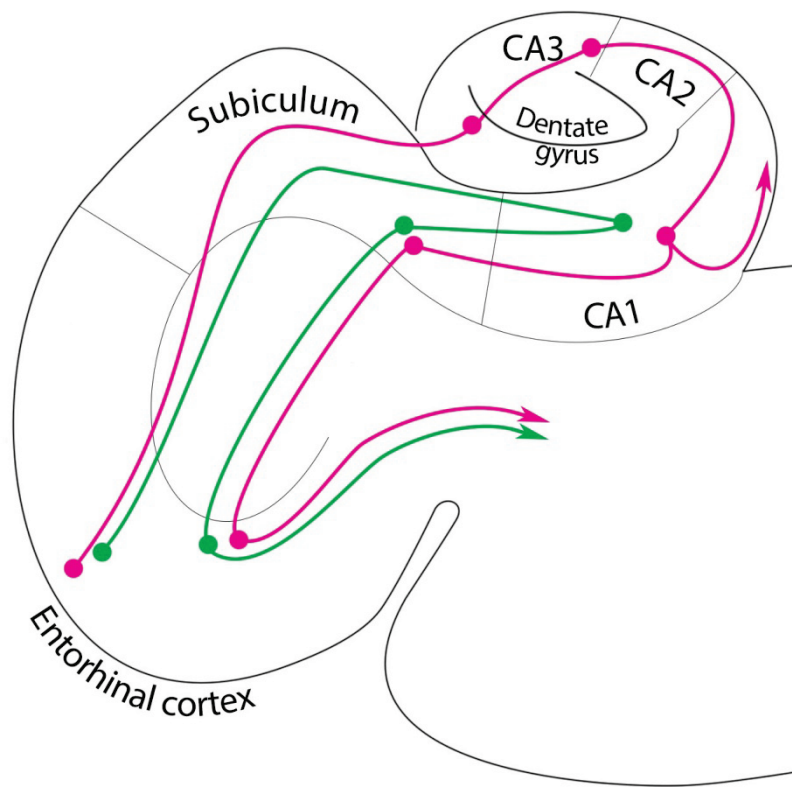


Figure 5.1 Hippocampal neural circuits. Illustration of the polysynaptic (pink) and direct (green) pathways in hippocampus. Based on circuitry described by Duvernoy et al. (2013).

As mentioned earlier, MRI studies have shown that the hippocampus of patients with OSA tends to be smaller than in age-matched controls. This decreased volume can be due to the loss of grey or white matter. Decreased integrity of the white matter is seen in the hippocampus, parahippocampal gyrus and temporal lobe of OSA patients compared to age-matched controls (H. L. Chen et al., 2015; Macey et al., 2008; Tummala, Roy, et al., 2016). This loss is thought to be the result of hypoxia rather than sleep fragmentation. Indeed, whole brain ischaemia causes demyelination (Y. Chen et al., 2013). Additionally, animal studies have shown that exposure to IH causes hypomyelination and reduces the expression of myelin-associated proteins in the cerebral cortex (L. J. Kim et al., 2015; Veasey et al., 2013). However, neither of these studies investigated the hippocampus.

Interestingly, hippocampal degeneration and memory impairments are common features of AD, a neurodegenerative dementia occurring in old age. The two neuropathological hallmarks of AD are the deposition of extracellular A β plaques and intraneuronal NFTs in the hippocampus. Further, there is significant loss of grey matter in the hippocampus, as evidenced by neuropathological studies of autopsy brain tissue from AD patients (Kril et al., 2002; West et al., 1994). Specific regions within the hippocampus that undergo loss of grey matter include the CA1, subiculum and entorhinal cortex (Gómez-Isla et al., 1996; Kril et al., 2004). While some of the neuronal loss is associated with the development of NFTs, other loss appears to be independent of NFTs (Gómez-Isla et al., 1997). Recently, with advances in the resolution of neuroimaging, volumetric losses have been confirmed in patients living with AD in the entorhinal cortex, CA1, CA2, CA3 and CA4 regions of the hippocampus (Delli Pizzi et al., 2016; Wisse et al., 2014). The most consistent difference in AD patients is a reduced CA1 volume compared to that in healthy elderly controls (de Flores, La Joie, & Chételat, 2015; Wolf, Fischer, de Flores, Chételat, & Fellgiebel, 2015). Histology studies from autopsy tissue show that white matter deteriorates in brain tissue from AD patients (Agosta et al., 2011; Pievani et al., 2010), including in the hippocampus and adjacent cortex (Salat et al., 2010), with all components of the white matter (myelin, oligodendrocytes and axons), showing signs of degeneration (Englund & Brun, 1990; Ihara et al., 2010). It is thought that myelin deterioration occurs prior to axonal damage, early in the pathological process of AD (Bartzokis, 2011; Englund & Brun, 1990).

OSA stands out as a potential contributor to the demyelination that occurs during the prodromal stage of AD. As noted above, OSA and IH are associated with injury to the cerebral white matter, and there is also a strong comorbidity between OSA and AD, with the presence of OSA in midlife being associated with the development of AD/dementia in later life (Chang et al., 2013; Yaffe et al., 2011). Furthermore, the prevalence of OSA in patients with AD/dementia is more than double the prevalence of OSA in the general elderly population (Gehrman et al., 2003; K. M. Rose et al., 2011; Young, Peppard, et al., 2002), and people with AD have a 5-fold increased risk of also having OSA (Emamian et al., 2016).

In OSA patients, treatment with CPAP is effective at eliminating hypoxic episodes and sleep disruption (Sullivan et al., 1981). Restoring proper oxygenation to the brain via CPAP can alleviate the cognitive symptoms associated with OSA (Canessa et al., 2011), but it is not consistently effective at restoring all of the affected cognitive domains (Lau et al., 2010). Neuroimaging research suggests that there are regional differences in the effectiveness of

CPAP treatment to reverse the volumetric loss and white matter damage seen in OSA (Canessa et al., 2011; Castronovo et al., 2014; H. Kim et al., 2016), which may depend on the capacity of the associated brain region to repair itself. For example, CPAP was shown to increase the volume of the dentate gyrus of OSA patients (H. Kim et al., 2016). Those authors suggested that neurogenesis was the cause of the volume increase observed, as the dentate gyrus is a known site of neurogenesis in the adult brain.

MRI measurements generally correlate well with cortical thickness measurements determined by histological methods (Kolasinski et al., 2012); however the large confidence intervals suggest that the method would be unreliable to estimate the thickness of individual cortical layers (Cardinale et al., 2014). Given that differences in cell layers can be indicative of specific neural circuitry, it is important to be able to determine the thickness of cortical layers individually. Histological measurements are commonly utilised for determining regional grey matter atrophy and neuronal loss in cases of epilepsy and hippocampal sclerosis (Reeves et al., 2016; Thom et al., 2012) and can be used to differentiate specific cell layers (Williams et al., 2013). Myelin is a key component of white matter and although changes in white matter can be determined through diffusion tensor MRI, the specific components of white matter (myelin, oligodendrocytes and axons) cannot be reliably differentiated (D. K. Jones, Knösche, & Turner, 2013). The immunohistochemical detection of myelin-associated proteins is commonly used to identify the location of demyelinating lesions in multiple sclerosis brains (Kuhlmann et al., 2017) and histopathological quantification of myelin loss has been used to investigate white matter injury in neurodegenerative diseases, including AD (Englund & Brun, 1990; Ihara et al., 2010; Sjöbeck, Haglund, & Englund, 2005).

The present study has investigated archived autopsy brain tissue from patients with a medical history of OSA and CPAP use. The aim of the study was to investigate the cell layer thickness and extent of myelination in specific hippocampal regions, and to relate these measures to OSA severity and CPAP use.

5.2 Methods

5.2.1 Study sample

The study sample consisted of autopsy brain tissue from 32 patients in the Iceland cohort, as described in Chapter 2. Sections of hippocampal tissue were used in this study. One case was excluded due to a diagnosis of multiple sclerosis and one case was excluded from white matter analysis due to tissue damage. Six cases were excluded from entorhinal cortex analysis due to insufficient tissue being present, however the cases were used for the other hippocampal regions.

5.2.2 Tissue processing

Brain tissue that had been embedded in paraffin wax blocks was sectioned at 20µm on a microtome and then processed for histology or immunohistochemistry, as described in Chapter 2.

5.2.3 Cresyl violet histology

Sections were stained with cresyl violet in order to visualise cell bodies, as described in Chapter 2.

5.2.4 Immunohistochemistry

The immunohistochemistry protocol followed that previously described in Chapter 2. Briefly, after deparaffinisation, sections to be immunostained for myelin basic protein (MBP) underwent antigen retrieval for 40 min at 80°C in EDTA buffer, prepared as previously described (Syrbu & Cohen, 2011). After blocking of non-specific antigenic sites, sections were incubated with primary antibody for anti-MBP (Abcam, ab7349) diluted at 1:1000 for 18 h at room temperature. Following this, sections were incubated with an appropriate secondary antibody for 6 h, and then processed as previously described (Chapter 2).

5.2.5 Neuronal counts

Sections stained with cresyl violet were used to estimate the number of neurons present in the pyramidal cell layer of each of the four regions of the hippocampus; CA1-4. Due to the very limited amount of tissue available, three-dimensional stereological estimates of total neuronal numbers could not be conducted. Instead, image-analysis was used to provide estimates of neuronal numbers from photomicrographs of defined regions. Photomicrographs were taken at 200X magnification (1200 x 1600 pixels) converted to greyscale and auto-contrasted. Based on careful histological examination at high magnification, it was determined that most objects with an area greater than 200 pixels² in size were neurons. Based on this criterion,

Olympus CellSens software was used to estimate the number of neurons in an image. Cell bodies that were bisected by the left hand or top margins of the micrograph were not included in the counts. Each image was visually examined to ensure that all of the obvious neurons had been selected, and no other cell types or other features had been included.

5.2.6 Hippocampus size measurements

Tissue sections stained with cresyl violet were also used for measurements of cell layer thickness and area. Three different parts of a section containing the hippocampus and parahippocampal gyrus were measured: the dentate gyrus, CA1 and entorhinal cortex (Figure 5.2A). Photomicrographs of the dentate gyrus and CA1 were taken at 40X magnification, entorhinal cortex micrographs were taken at 64X magnification. Olympus CellSens software was used to measure distances and areas of specific regions, as detailed below for each region.

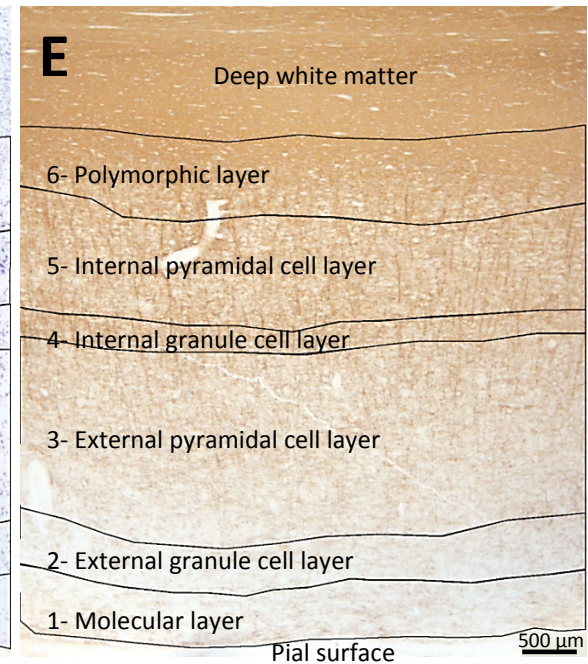
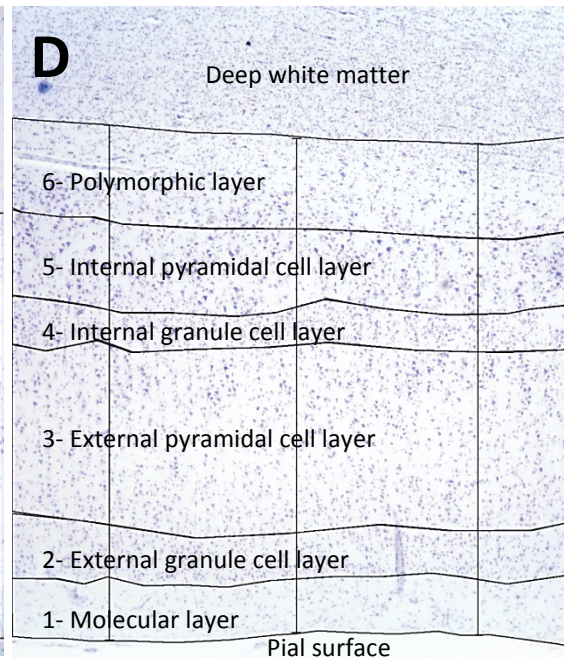
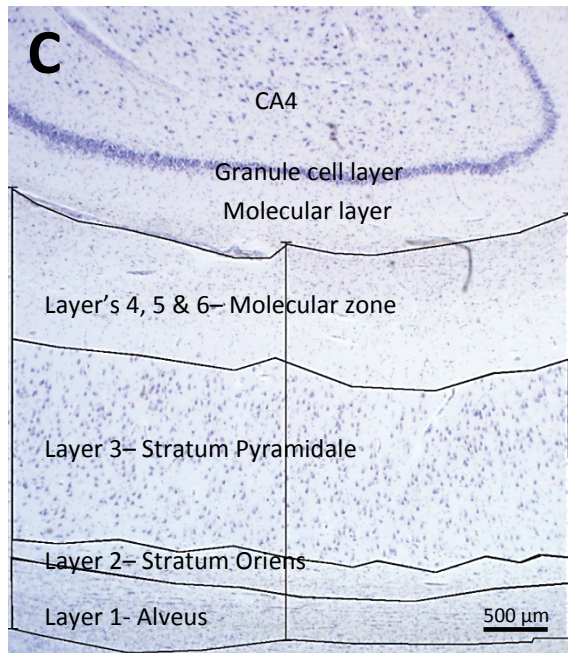
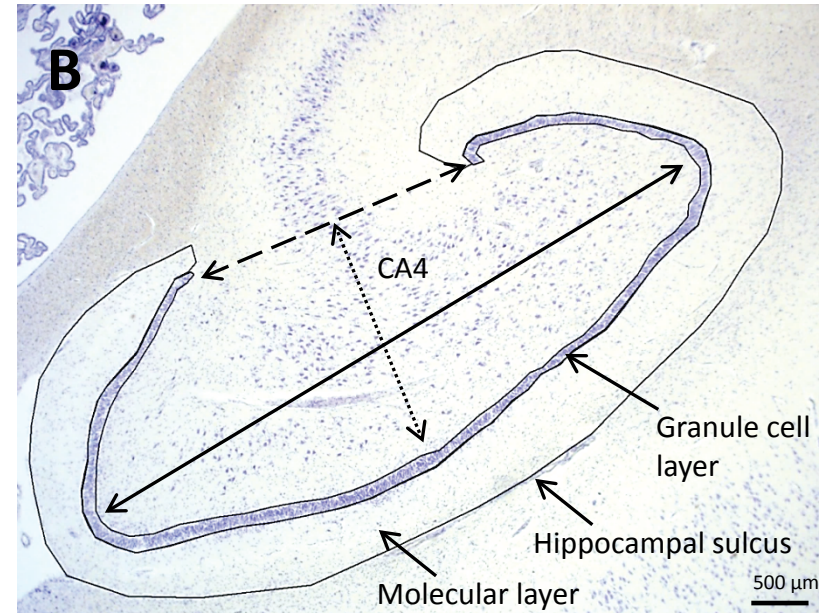
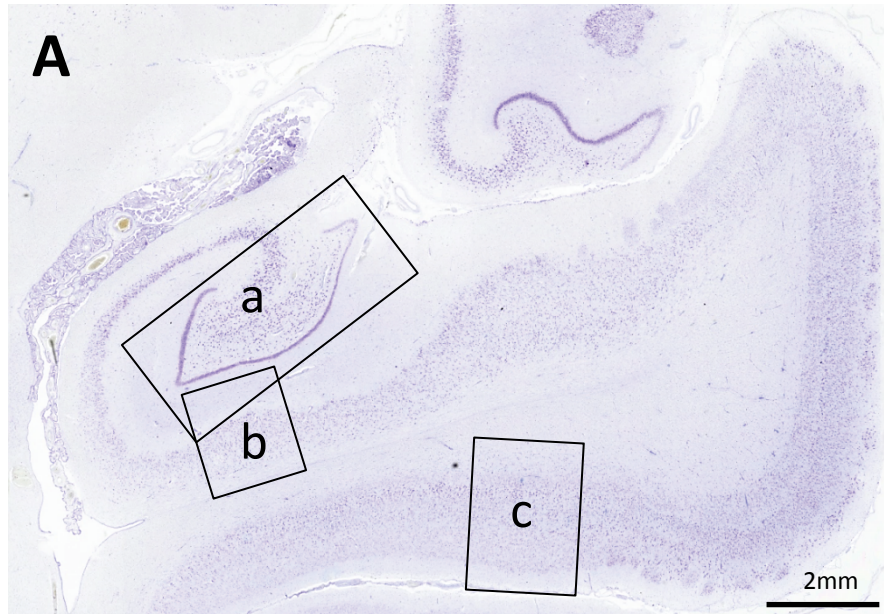


Figure 5.2 Cresyl violet and MBP images of hippocampal regions. Low magnification cresyl violet (A-D) and MBP (E) images of hippocampus (A) with boxes indicating the areas sampled for the dentate gyrus (a), CA1 (b) and entorhinal cortex (c). Dentate gyrus (B) including measurements taken for the hilus length (solid arrow), hilus opening (dashed arrow) and the depth of the hilus perpendicular to the hilus opening (dotted arrow). CA1 (C) indicating the 4 different layers measured. Entorhinal cortex (D & E) indicating the 6 cortical layers measured and the deep white matter. Scale bar in E applies to panel D.

Features measured in the dentate gyrus were: the width of the opening of the hilus, the depth perpendicular to the opening of the hilus, the length of the hilus and the area of the molecular and granule cell layers (Figure 5.2B). The layers of the dentate gyrus were identified from histological features, as previously described (Duvernoy et al., 2013). The granule cell layer is prominent in cresyl violet-stained sections due to the dense clustering of cells. The molecular cell layer was defined as the region between the outer border of the granule cell layer and the hippocampal sulcus.

The total cortical thickness of the CA1 and entorhinal cortex was calculated as the mean of three measurements of the cortex from pia matter to the deep white matter on each micrograph. The boundary of each layer of the cortex was then estimated, from histological characteristics, such as the presence or absence of certain cell types (Duvernoy et al., 2013). The percentage area of each layer was calculated using the sum area of all cortical layers across the same lateral distance (width of the micrograph, 1200 pixels). The percentage area occupied by each layer was divided by the mean total cortical thickness that was initially recorded, in order to give measurements for the cortical thickness of each layer of cortex. Figure 5.2C and D illustrate the measurements taken from typical cresyl violet images at the CA1 and entorhinal cortex, respectively.

In the CA1, four cortical layers were measured (Figure 5.2C). Layer 1: the alveus, consists mostly of white matter and is directly underneath the pial surface. Layer 2: stratum oriens, consists of mostly basket cells and axons although there is less white matter than layer 1. Layer 3: stratum pyramidale, is the largest layer and contains pyramidal neurons. The

molecular zone is made up of layers 4 (stratum radiatum), 5 (stratum lacunosum) and 6 (stratum moleculare). These layers are notoriously difficult to distinguish from each other in the CA1 and for this reason are collectively referred to as the molecular zone (Duvernoy et al., 2013). In the present study, the terms ‘molecular zone’ and ‘layer 4 of CA1’ are used interchangeably.

In the entorhinal cortex, six cortical layers were measured as described by Vanderah, Gould, and Nolte (2016); layer 1 (molecular layer), layer 2 (external granule cell layer), layer 3 (external pyramidal cell layer), layer 4 (internal granule cell layer), layer 5 (internal pyramidal cell layer) and layer 6 (polymorphic layer). Layer 1 is directly beneath the pial surface; it contains mostly axons and few neuronal cell bodies. Layers 3 and 5 contain pyramidal neurons; layers 2 and 4 consist mainly of granule cells rather than pyramidal cells; while layer 6 contains modified pyramidal cells as well as many axons (Figure 5.2D & E).

5.2.7 Myelin basic protein quantification & analysis

Micrographs of MBP staining were taken at the same magnification and in the same regions as the cresyl violet-stained sections. These images were converted to greyscale and the mean grey intensity of the area of each cortical layer was measured. The area of the cortical layer was copied as a template from the cresyl violet image and pasted onto the MBP image. Minor adjustments were made to the area where the borders of the layers did not line up exactly with the cresyl violet image (Figure 5.2E). Measurements were also made of the mean grey intensity in the deep white matter of the entorhinal cortex. The deep white matter was defined as being below the boundary of layer 6 of the cortex. The area of this region was not defined individually; rather a standard area (0.34 mm^2) was used for all images to obtain a value for the mean grey intensity. The inverse mean grey intensity was used for all graphs, so that lower grey intensity values correspond to lighter staining, indicating decreased myelin content. The inverse mean grey intensity was calculated by subtracting the mean grey intensity from 256 (the maximum value of any grey scale image).

5.2.8 Statistical analysis

All statistical analysis was performed using the IBM SPSS. Regressions were performed between log ODI and the various hippocampal size measurements and the inverse myelin

basic protein staining. Similarly, regressions were performed when the sample was stratified by CPAP use.

5.3 Results

5.3.1 Descriptive statistics

Descriptive statistics for the sample are given in Table 5.1. A significant correlation was found between ODI and age, $r=0.421$, $p=0.016$. No other significant correlations were found between age, ODI, BMI or interval from diagnosis to death. CPAP users and non-users were compared using two-tailed student t-tests. CPAP users were found to have a significantly higher BMI than CPAP non-users, $t(26)=3.19$, $p=0.004$. No differences were found for age, ODI or interval from diagnosis to death.

Table 5.1 Descriptive statistics for total sample and separated by CPAP use. Mean \pm standard deviation

	Total	CPAP users	CPAP non-users	Significance CPAP users vs. non-users
n	32	18	14	
Gender	F=17, M=15	F=12, M=6	F=5, M=9	
Age at death (years)	67.0 \pm 11.0	69.9 \pm 11.1	63.3 \pm 10.2	p=0.096
BMI kg/m²	29.9 \pm 5.9	32.6 \pm 5.8	26.3 \pm 4.0	p=0.004*
	n=28	n=16	n=12	
Time from OSA diagnosis to death (years)	7.6 \pm 5.9	7.1 \pm 6.2	8.3 \pm 5.6	p=0.563
ODI (events/h)	26.5 \pm 21.4	32.0 \pm 18.8	19.5 \pm 23.2	p=0.104

*p<0.05 CPAP users compared to CPAP non-users

5.3.2 Hippocampus size

In the dentate gyrus, there was a significant negative relationship between the length of the hilus and log ODI, $r^2=0.129$, $p=0.044$ (Figure 5.3A). No relationship was seen for the width of the opening of the hilus or the depth perpendicular to the opening (Figure 5.3B & C).

When the sample was divided into CPAP users and CPAP non-users, no significant relationships were found (Figure 5.4A, B & C). No relationship was seen between log ODI and the area of the granule cell layer. However, a significant negative relationship was found between log ODI and the area of the molecular layer of the dentate gyrus, $r^2=0.136$, $p=0.038$ (Figure 5.3D), indicating that the area of the molecular layer decreased with increasing ODI value. This relationship strengthened (higher r^2 value) when only CPAP non-users were considered, $r^2=0.352$, $p=0.025$, and did not change (to two decimal places) or reach significance when only CPAP users were considered, $r^2=0.138$, $p=0.129$ (Figure 5.4D). These results indicate that CPAP use may protect against shrinkage of the molecular layer of the

dentate. Figure 5.5A & B show representative micrographs of the dentate gyrus from patients with low and high ODI's respectively. In the patient with low ODI, the length of the hilus is longer and the area of the molecular layer is larger (Figure 5.5A), compared to the patient with high ODI (Figure 5.5B).

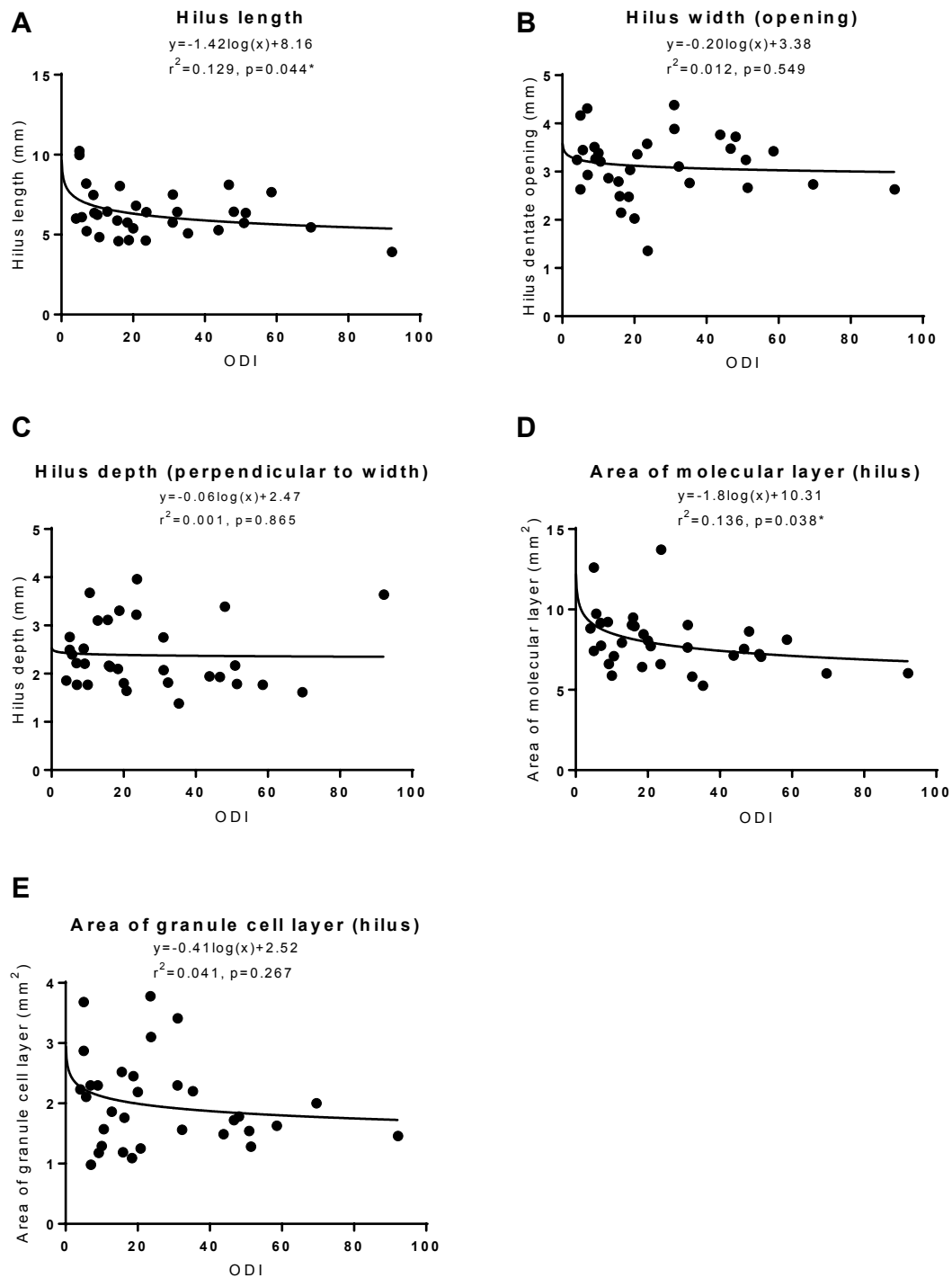


Figure 5.3 Dentate gyrus size measurements regressed against OSA severity (ODI). Total length of the hilus (A), width of the opening of the hilus (B), hilus depth perpendicular to the width of the opening (C), area of the molecular layer (D) and granule cell layer (E) of the dentate (hilus) of the hippocampus.* indicates $p < 0.05$.

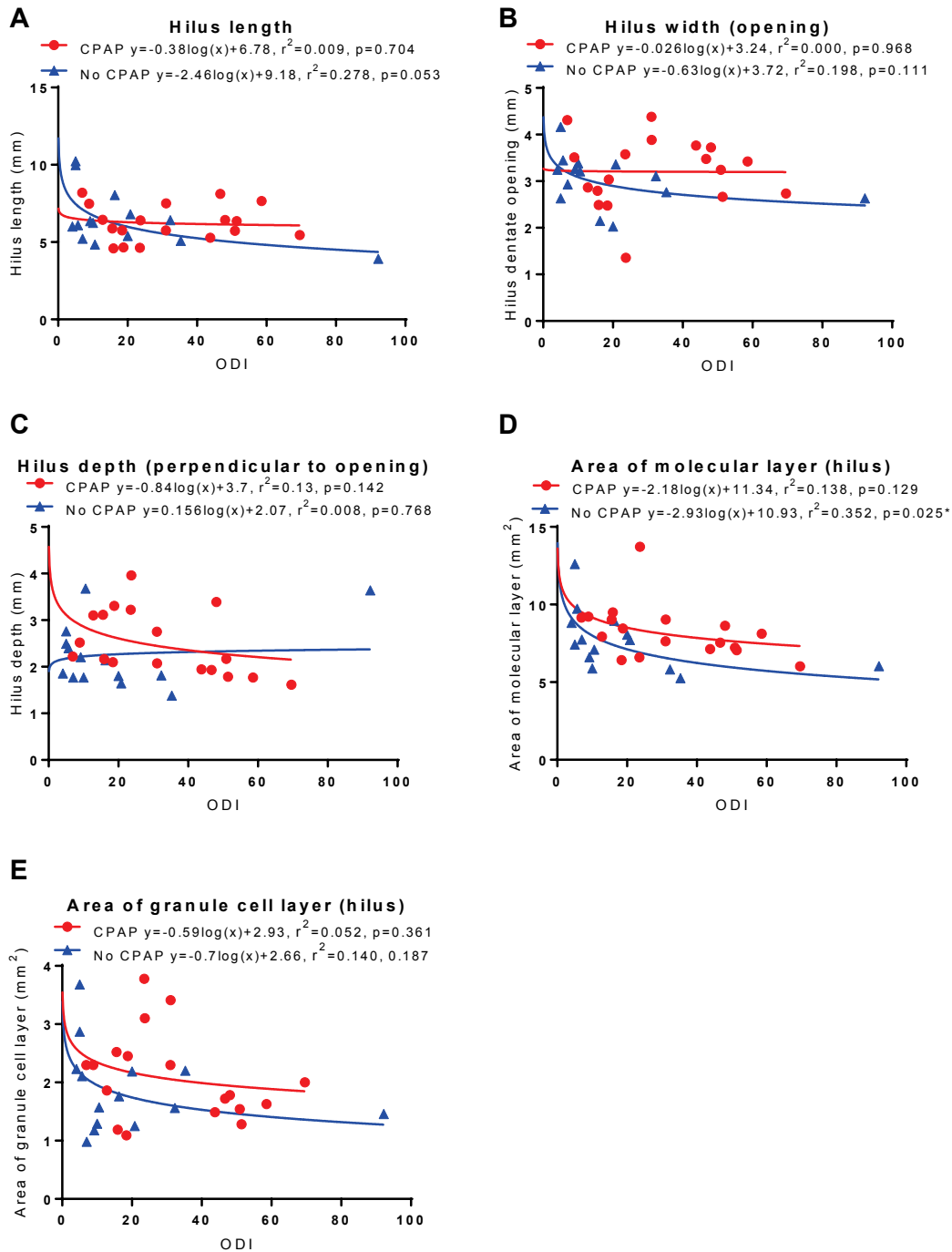


Figure 5.4 Dentate gyrus size measurements regressed against OSA severity (ODI), separated by CPAP use. Total length of the hilus (A), width of the opening of the hilus (B), hilus depth perpendicular to the width of the opening (C), area of the molecular layer (D) and granule cell layer (E) of the dentate (hilus) of the hippocampus. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p < 0.05$.

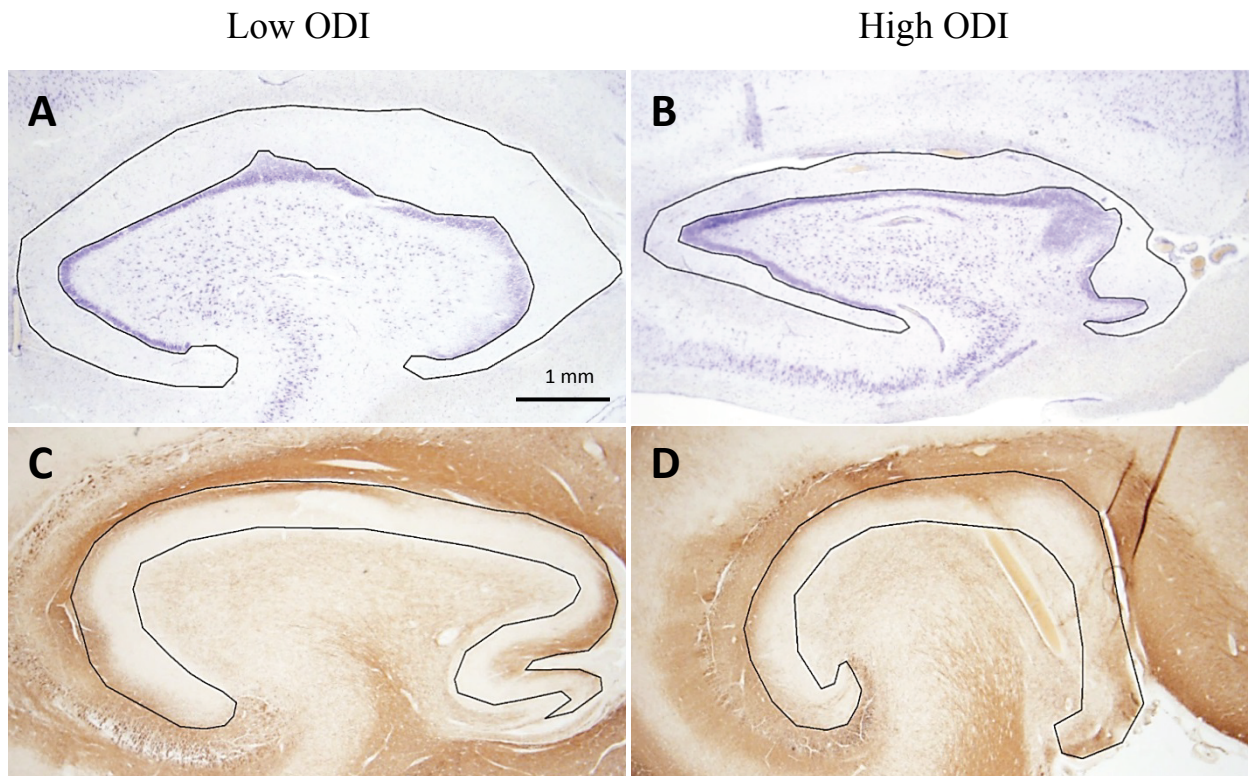


Figure 5.5 Micrographs of the dentate gyrus. Sections stained for cresyl violet (A & B) and MBP immunohistochemistry (C & D) from a patient with low (A & C) and high (B & D) ODI score. Notice the smaller area of the molecular layer in the top right image compared to the top left and the similarity of staining between the bottom two images in this layer. Scale bar =1mm and applies to all panels.

There was a significant negative correlation between log ODI and total cortical thickness of the CA1, $r^2=0.135$, $p=0.039$, but not for the thickness of the entorhinal cortex (Figure 5.6A & B). Further, when the sample was separated into CPAP users and non-users (Figure 5.6C & D), the correlation in the CA1 strengthened for CPAP non-users (higher r^2 value compared to whole sample), $r^2=0.315$, $p=0.037$, and weakened in CPAP users (lower r^2 value compared to whole sample), $r^2=0.045$, $p=0.401$. Although there was no significant relationship for the total cortical thickness in the entorhinal cortex, when separated by CPAP use, the same trend was

seen as for the CA1. CPAP non-users had a significant negative relationship between the thickness of the entorhinal cortex and log ODI, $r^2=0.401$, $p=0.049$, and no relationship was seen in CPAP users, $r^2=0.020$, $p=0.601$. These results suggest that CPAP non-users experience a decrease in cortical thickness of the CA1 and the entorhinal cortex with increasing ODI, whereas CPAP users do not.

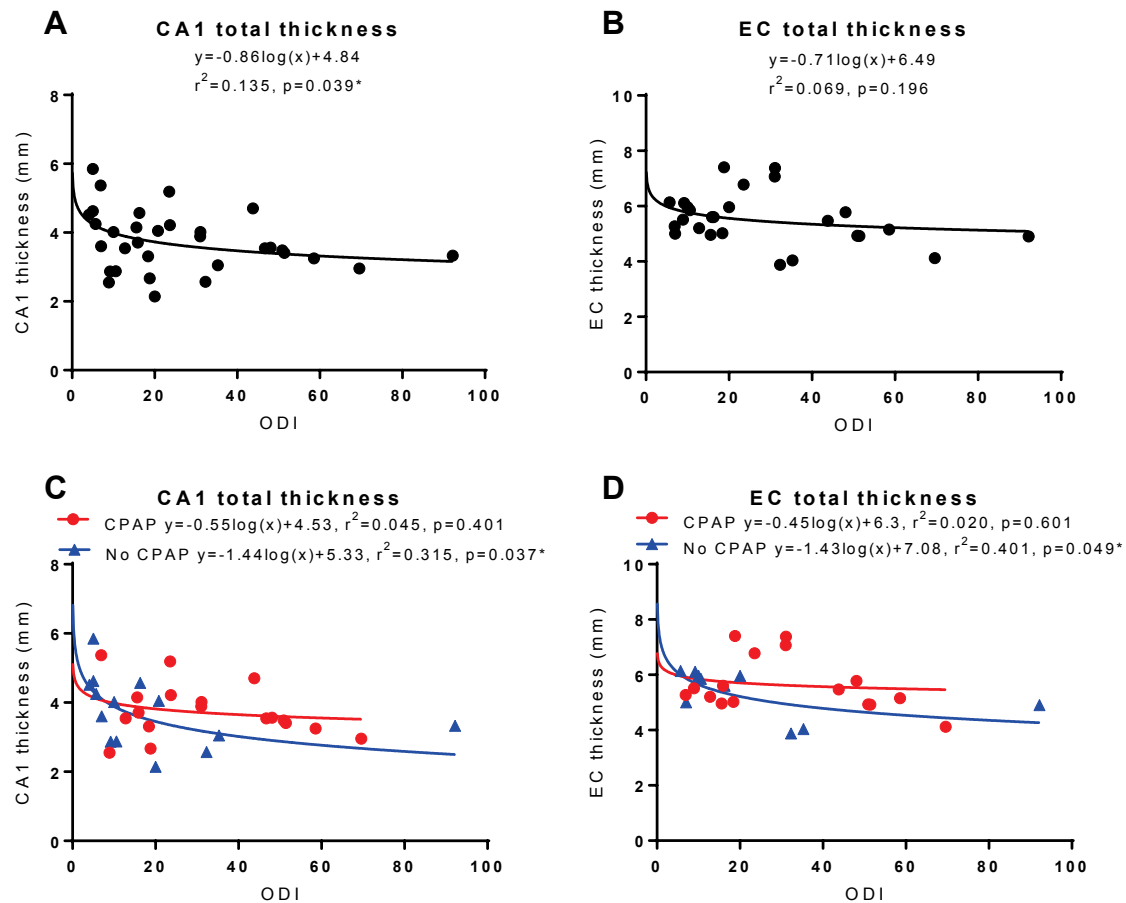


Figure 5.6 Overall thickness of CA1 and EC. Cortical thickness measurements in mm in the CA1 and EC regressed against OSA severity (ODI). CA1 (A & C) and EC (B & D) total thickness measurements (A & B) and separated by CPAP use (C & D). CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. EC: entorhinal cortex. * indicates $p < 0.05$.

In the CA1 region of the hippocampus, the thickness of each of the 4 cortical layers (layers 1, 2, 3 and the molecular zone comprised of layers 4-6) was measured individually. Significant negative relationships were found between layer thickness and log ODI in layer 1, $r^2=0.157$, $p=0.025$; layer 2, $r^2=0.255$, $p=0.003$; and layer 3, $r^2=0.185$, $p=0.014$; but not in the molecular zone (Figure 5.7). Figure 5.9 shows representative micrographs from patients with low ODI (Figure 5.9A) and high ODI scores (Figure 5.9B). The decrease in overall thickness can be seen; the distance from the top black line to the bottom black line is larger in the patient with less severe OSA (Figure 5.9A) compared to the patient with more severe OSA (Figure 5.9B). Specifically, there is decreased cortical thickness in layers 1-3 in the patient with more severe OSA. The sample was then separated into CPAP users and non-users (Figure 5.8). There was a strengthening of the relationship between log ODI and the CA1 layer thickness for CPAP non-users in layer 1, $r^2=0.375$, $p=0.02$; layer 2, $r^2=0.294$, $p=0.045$; and layer 3, $r^2=0.338$, $p=0.029$; no relationship was present among CPAP users. No relationship was seen in the CA1 layer 4.

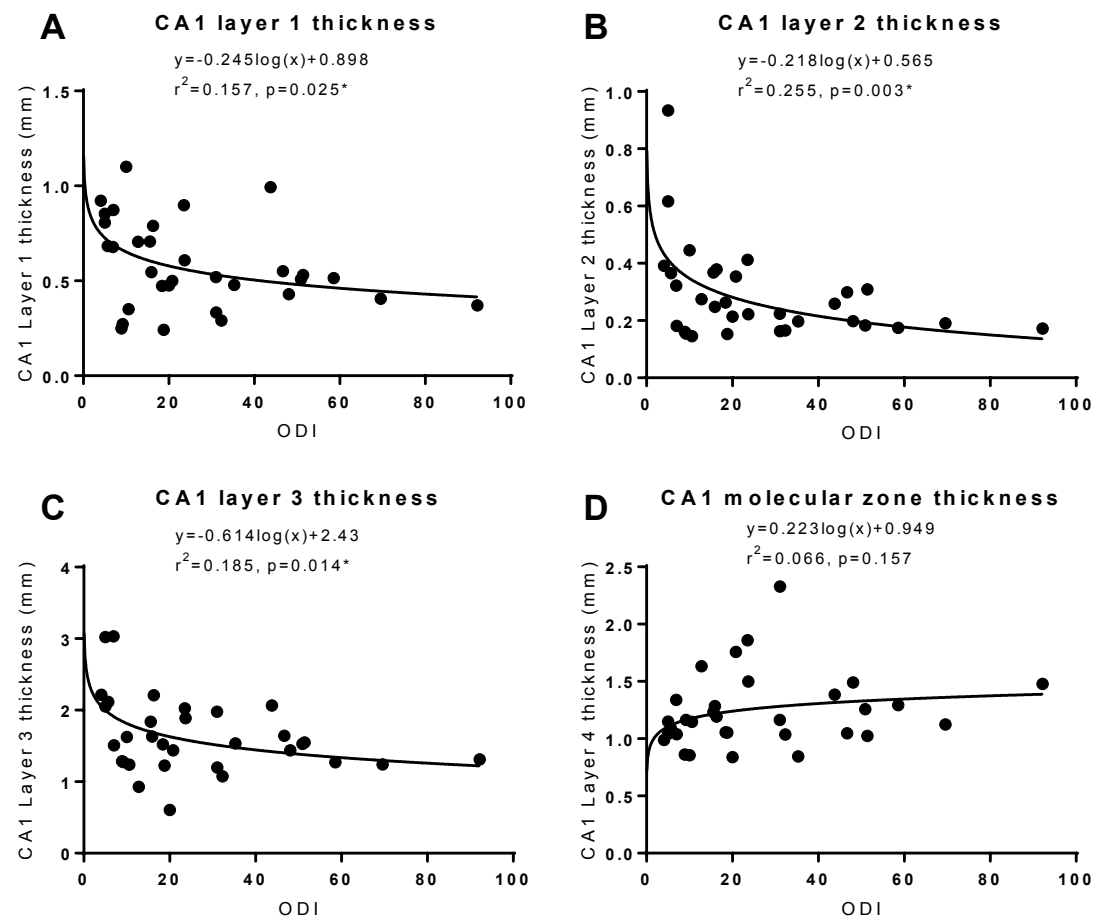


Figure 5.7 Cortical layer thickness of the CA1 of the hippocampus regressed against OSA severity (ODI). * indicates $p < 0.05$.

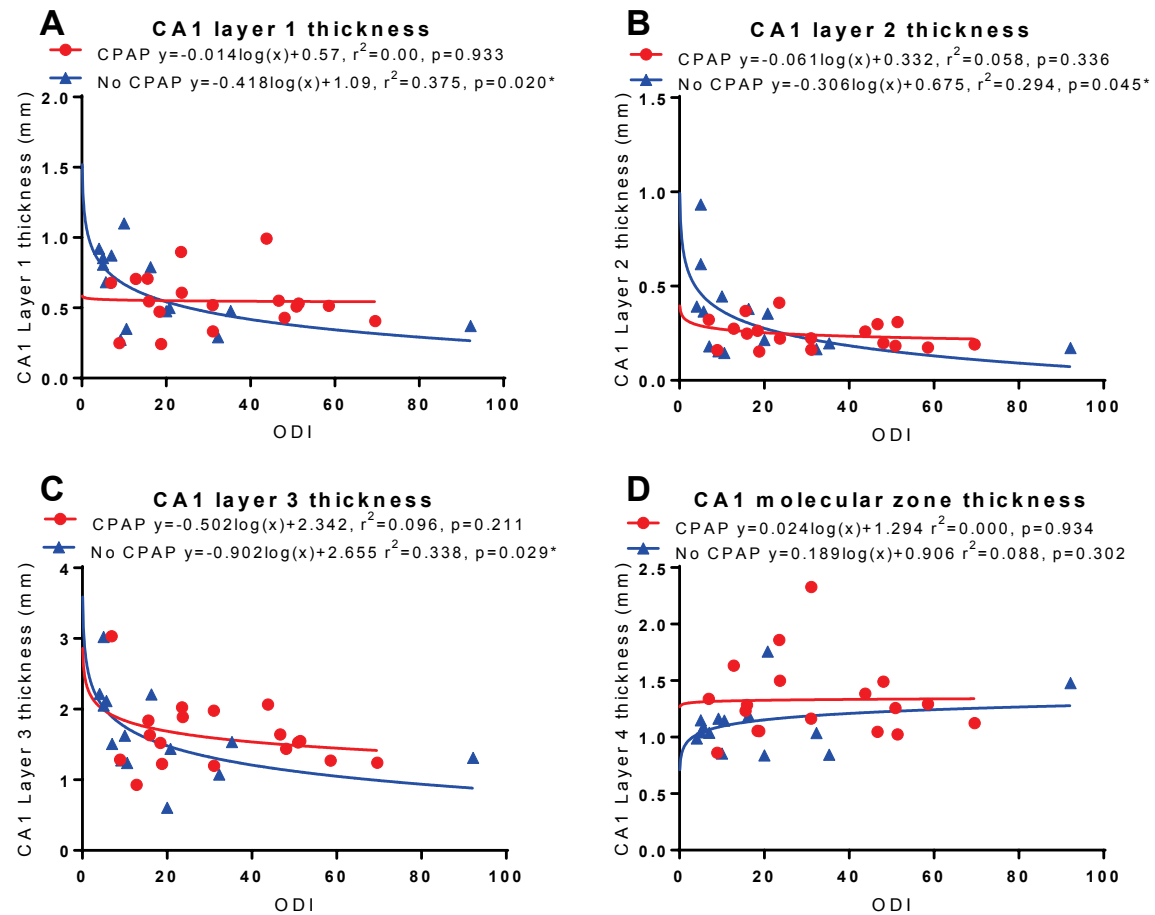
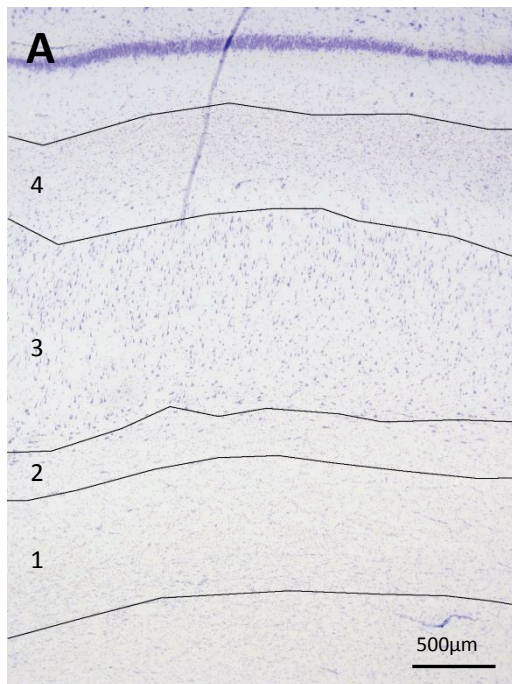


Figure 5.8 Cortical layer thickness of the CA1 regressed against OSA severity (ODI), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p < 0.05$.

Low ODI



High ODI

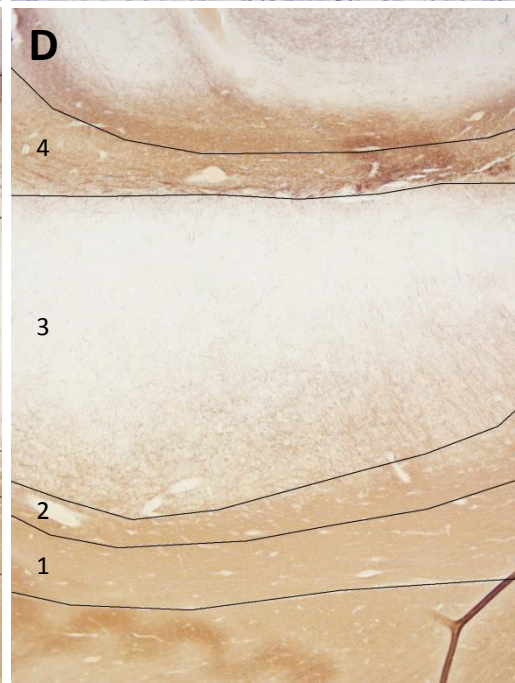
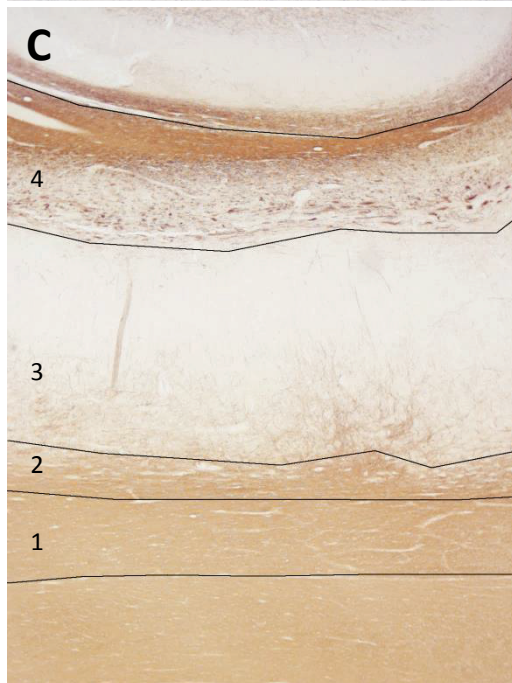
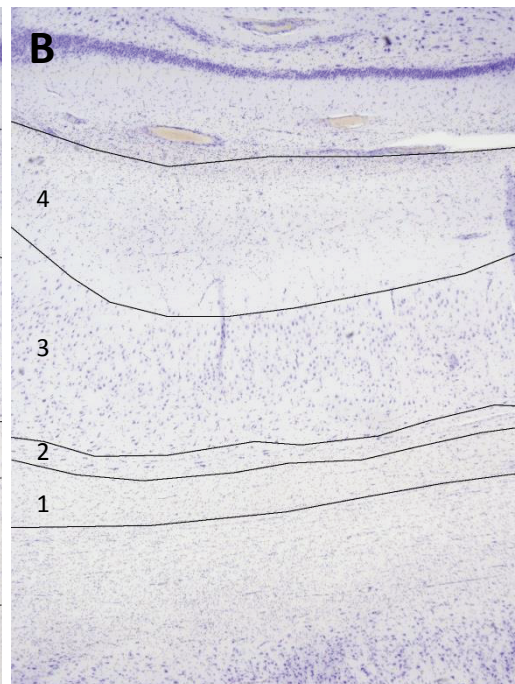


Figure 5.9 Micrographs of the CA1. Sections stained for cresyl violet (A & B) and MBP immunohistochemistry (C & D) from a patient with a low ODI score (A & C) and a high ODI score (B & D). Notice the thinner layers (1, 2 & 3) in the top right image compared to the top left and the similarity of the bottom right image compared to the bottom left. Scale bar = 500µm and applies to all panels.

5.3.3 Neuronal counts in hippocampus

Neuron numbers were estimated in the pyramidal cell layer of the CA1-CA4 regions of the hippocampus (Figure 5.10). No significant relationships were seen between neuron estimates and ODI in any of the four regions, even when separated into CPAP users and non-users (Figure 5.11).

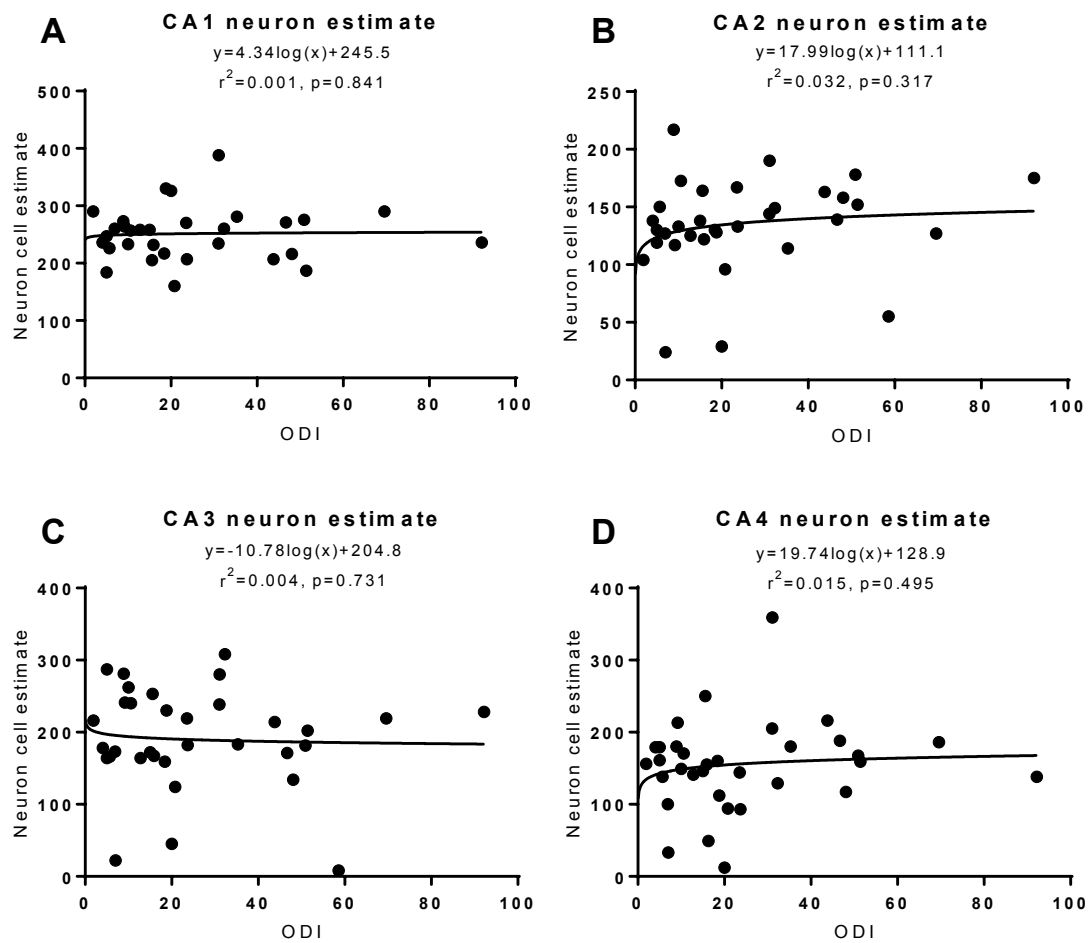


Figure 5.10 Estimates of neuron number in sections of the pyramidal cell layer (layer 3) in each of the four hippocampal regions, regressed against OSA severity (ODI).

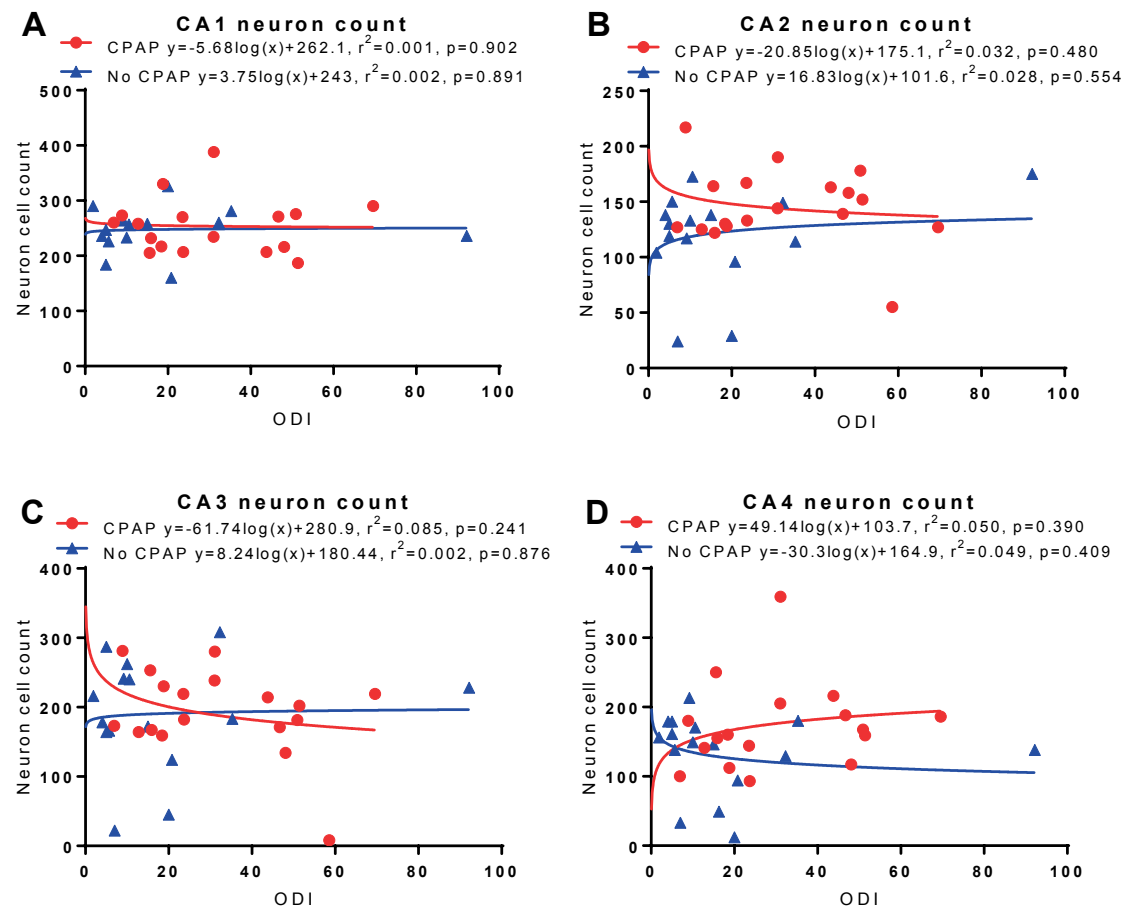


Figure 5.11 Estimates of neuron number in sections of the pyramidal cell layer (layer 3) in each of the four hippocampal regions, regressed against OSA severity (ODI), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles.

5.3.4 Entorhinal cortex size

Layers of the entorhinal cortex were measured individually to determine if there were any changes in the thickness of the different cortical layers (Figure 5.12, 5.13 & 5.14). A significant negative correlation between log ODI and cortical layer thickness was found in layer 1, $r^2 = 0.186$, $p = 0.028$, and a trend was seen in layer 3, $r^2 = 0.124$, $p = 0.078$ (Figure 5.12A & C). No significant relationships were seen for layers 2, 4, 5 or 6 (Figure 5.12B, D, E & F). When the sample was separated into CPAP users and non-users (Figure 5.13), there were

stronger negative correlations for CPAP non-users between log ODI and layer thickness in layers 1, 2 and 3. However, only the layer 3 correlation reached significance, $r^2=0.617$, $p=0.007$. The correlations for layer 1, $r^2=0.391$, $p=0.053$, and layer 2, $r^2=0.4$, $p=0.05$, approached statistical significance. Figure 5.14 shows representative micrographs from a patient with low ODI score (Figure 5.14A) and high ODI score (Figure 5.14B). No difference in cortical thickness is evident in layers 2, 3, 4, 5, or 6. Layer 1 is thinner in the patient with a high ODI (Figure 5.14B). Table 5.2 summarises the significant findings from the preceding sections.

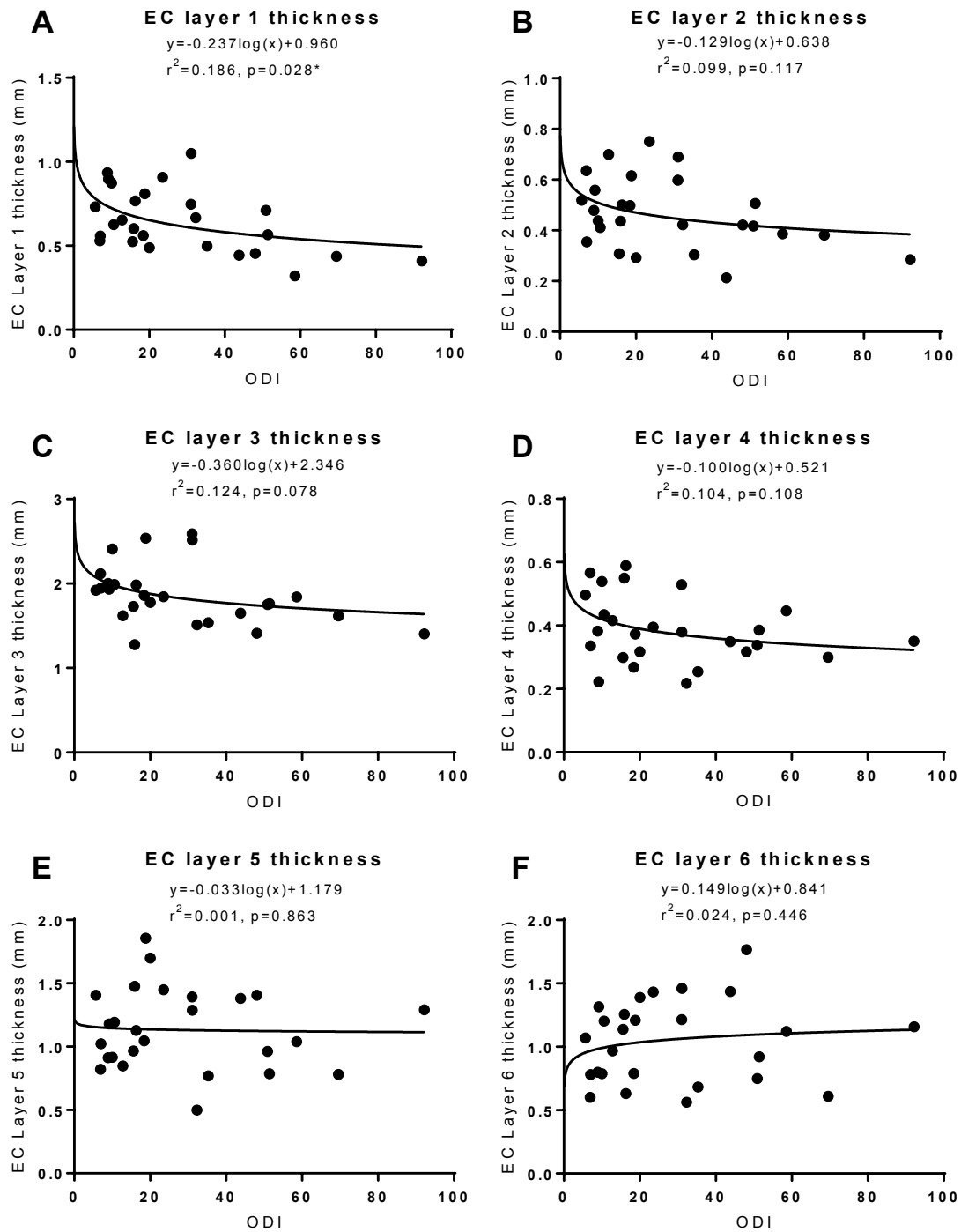


Figure 5.12 Thickness of the layers of the EC regressed against OSA severity (ODI). EC: entorhinal cortex. * indicates $p < 0.05$.

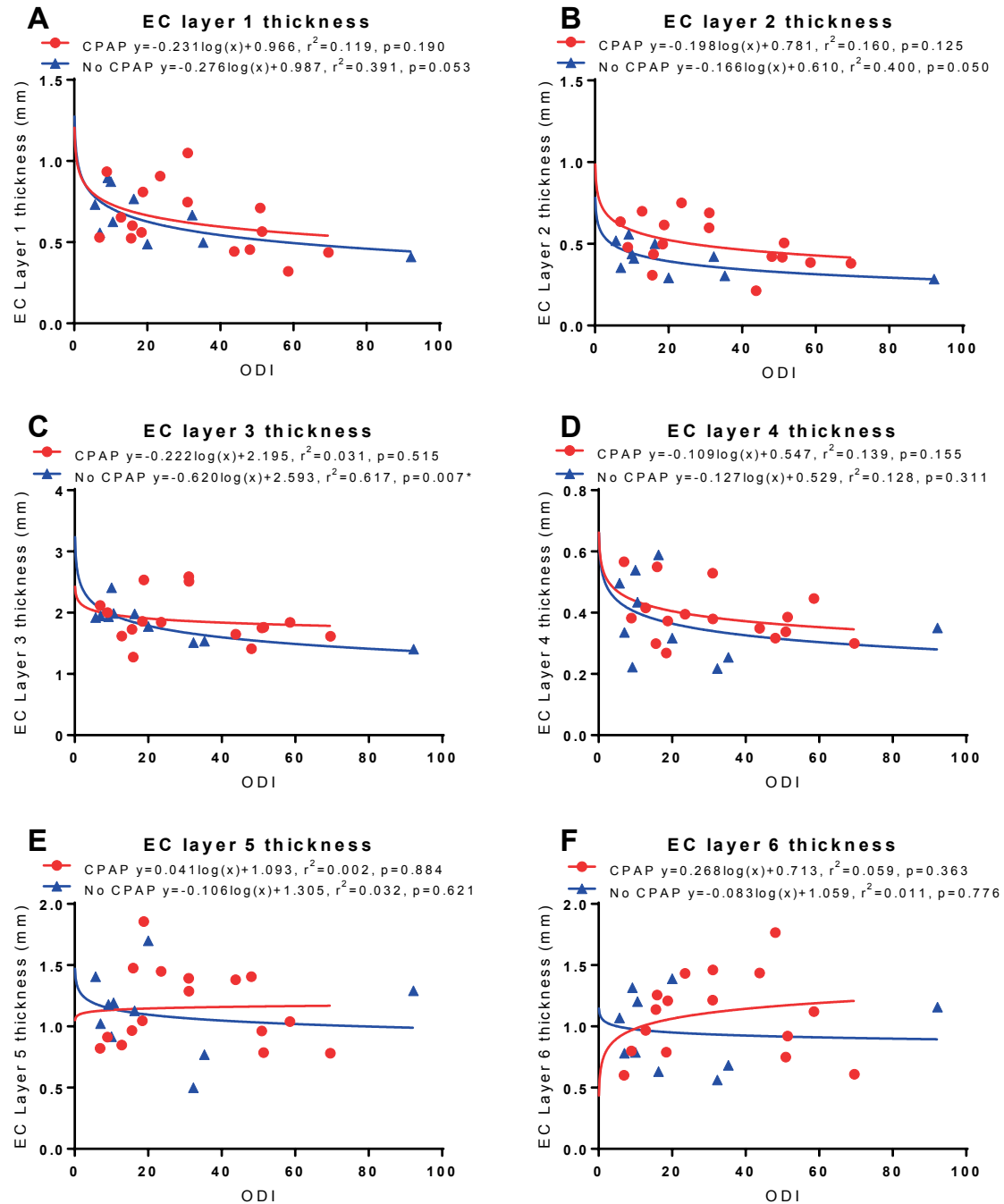
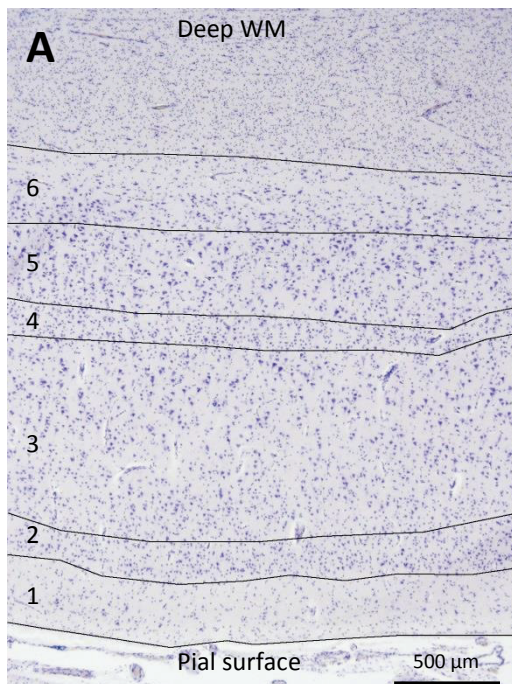


Figure 5.13 Thickness of the layers of the EC regressed against OSA severity (ODI), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. EC; entorhinal cortex. * indicates $p < 0.05$.

Low ODI



High ODI

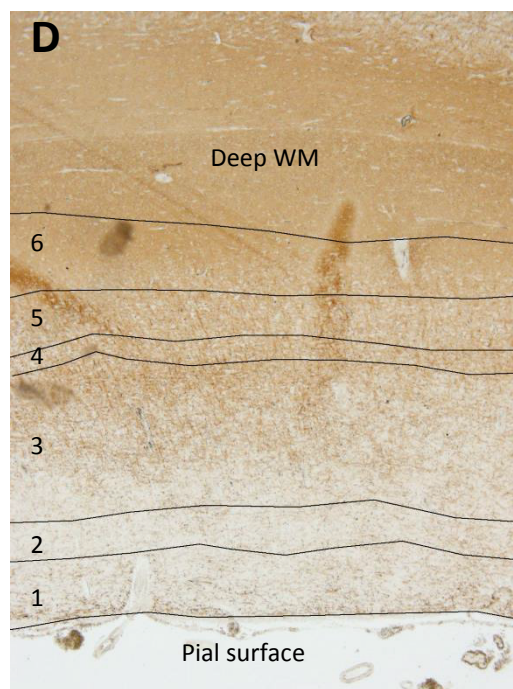
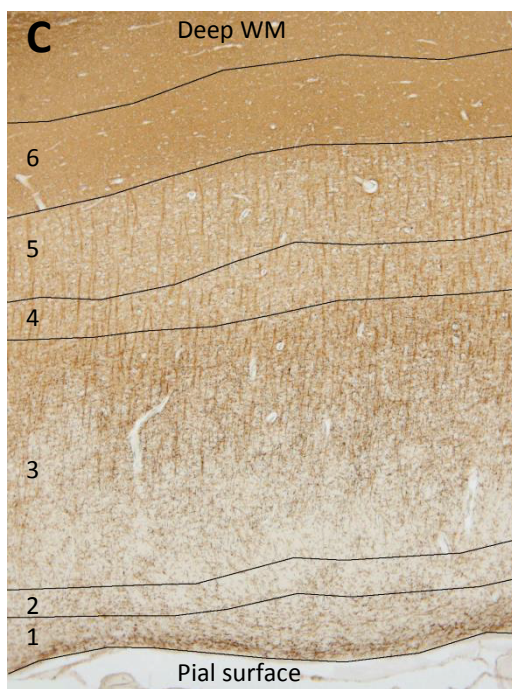
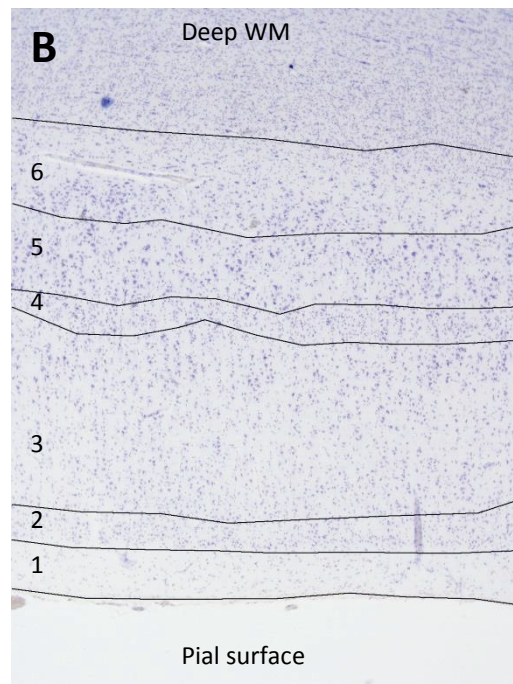


Figure 5.14 Micrographs of the EC. Sections stained for cresyl violet (A & B) and MBP immunohistochemistry (C & D) from a patient with a low ODI score (A & C) and a high ODI score (B & D). Notice layer 1 is thinner in the top right image compared to the top left and the lighter staining of layer 6 and the deep WM in the bottom right image compared to the bottom left. EC: entorhinal cortex; WM: white matter. Scale bar = 500µm and applies to all panels.

Table 5.2 Summary table of significant changes in hippocampal size

	Direction and magnitude of change as a function of increasing ODI	Effect of CPAP use (size of r^2)	Effect of not using CPAP (size of r^2)
CA4 hilus length	↓	↓	↓
CA4 area of molecular layer	↓	↓	↓
CA1 total thickness	↓	↓	↓
CA1 layer 1 thickness	↓	↓	↓
CA1 layer 2 thickness	↓	↓	↓
CA1 layer 3 thickness	↓	↓	↓
EC total thickness*	↓	↓	↓
EC layer 1 thickness	↓	↓	↓
EC layer 3 thickness#	↓	↓	↓

↓ = smaller reduction, ↓ = moderate reduction, ↓ = larger reduction, * = no significant relationship for total sample; # = trend for a relationship for total sample

5.3.5 Myelin staining intensity

The inverse of the mean grey intensity of MBP staining was used to approximate the amount of myelin, where higher values represent more intense staining (more myelin) and lower values represent less intense staining (less myelin). In the dentate gyrus, no relationship was

seen between MBP staining intensity and ODI in the granule cell and molecular layers (Figure 5.15A & B; Figure 5.5C & D). Within the molecular and granule cell layers of the dentate there is no difference in the intensity of myelin staining between a patient with low ODI (Figure 5.5C) and a patient with high ODI (Figure 5.5D). When separated by CPAP use (Figure 5.15C and D), CPAP non-users showed stronger negative relationships between ODI and MBP staining than the CPAP users in both the molecular and granule cell layers; however neither relationship was significant.

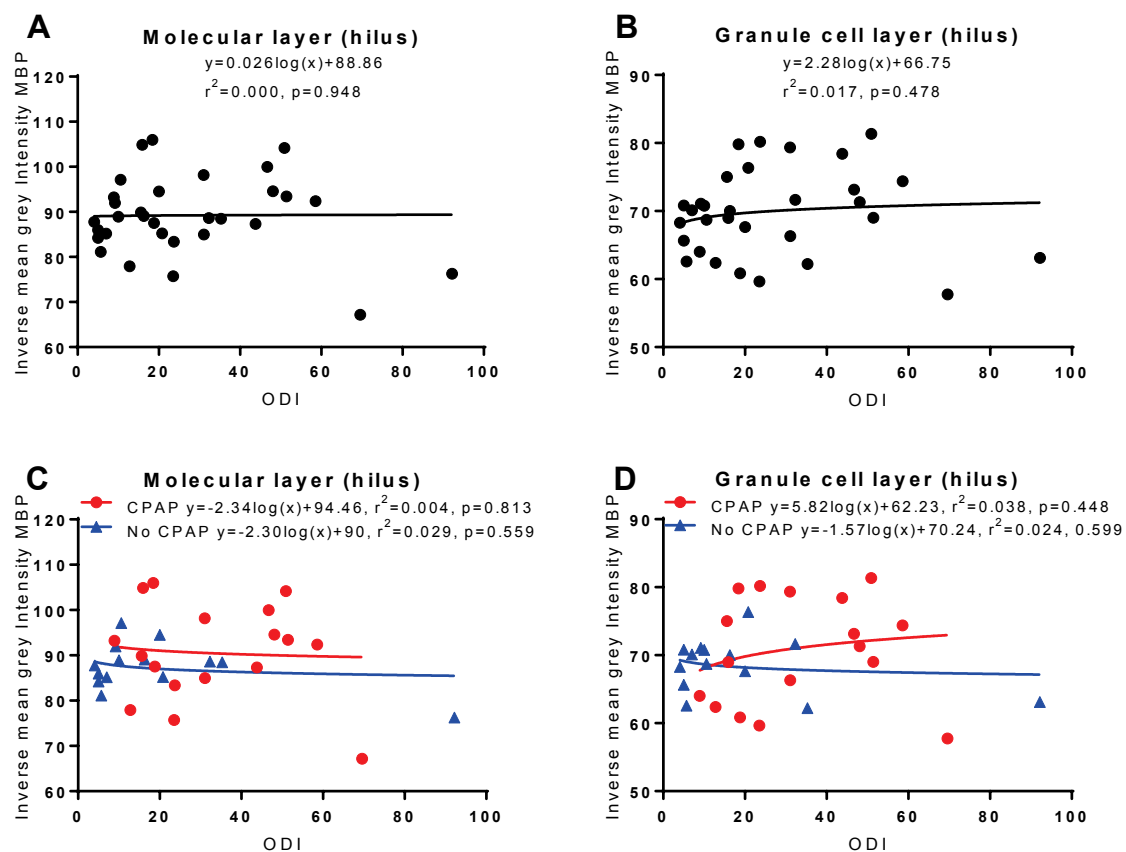


Figure 5.15 The inverse mean grey intensity of MBP staining regressed against OSA severity (ODI), for the molecular layer (A & C) and the granule cell layer (B & D) of the hilus. All data are shown in the top panels (A & B), and are separated by CPAP use in the lower panels (C & D). CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles.

In the CA1 region of the hippocampus, no significant relationships were found between ODI and MBP staining intensity in any layers (Figure 5.16; Figure 5.9C & D). However, when stratified by CPAP use there was a significant negative relationship between ODI and MBP staining in layer 2 for CPAP users, $r^2=0.274$, $p=0.031$, (Figure 5.17B). No significant relationship was seen for non-CPAP users, or any other layer of CA1.

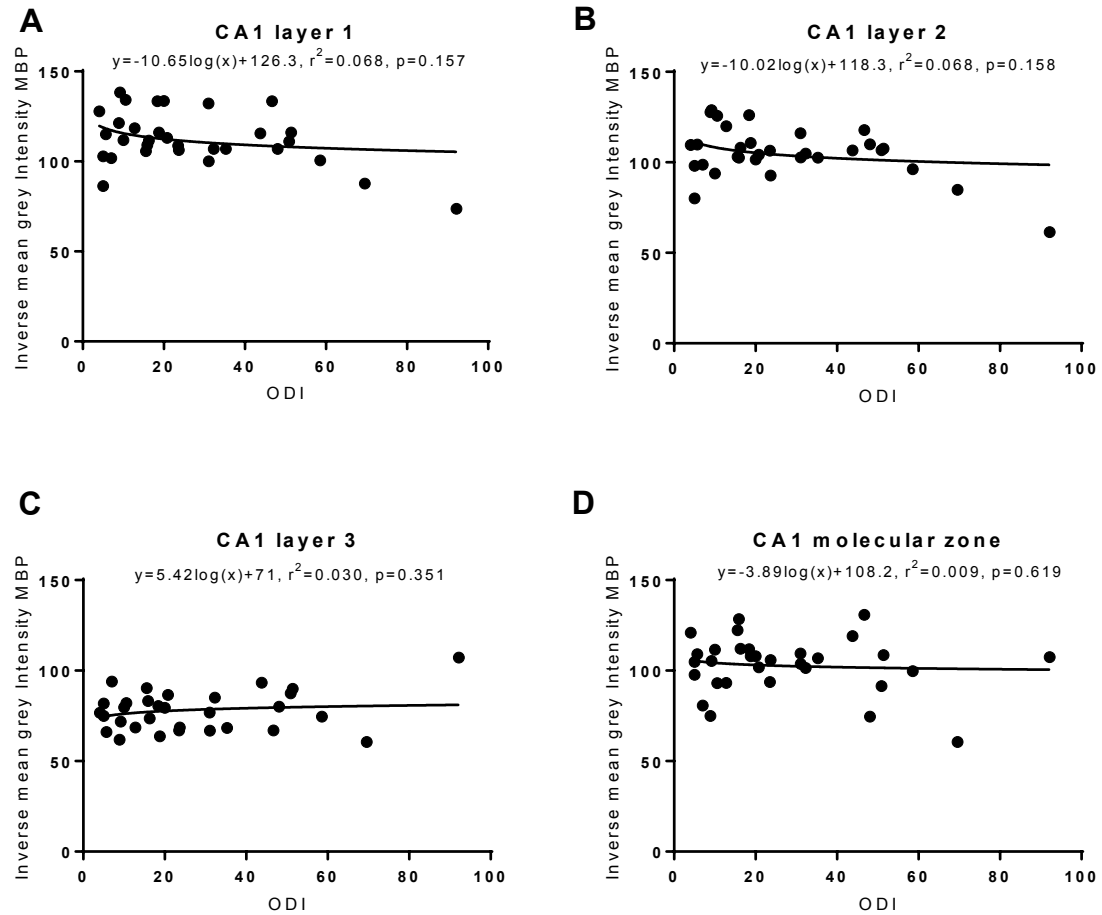


Figure 5.16 The inverse mean grey intensity of MBP staining regressed against OSA severity (ODI), for the CA1 separated by layer (A-D). * indicates $p < 0.05$.

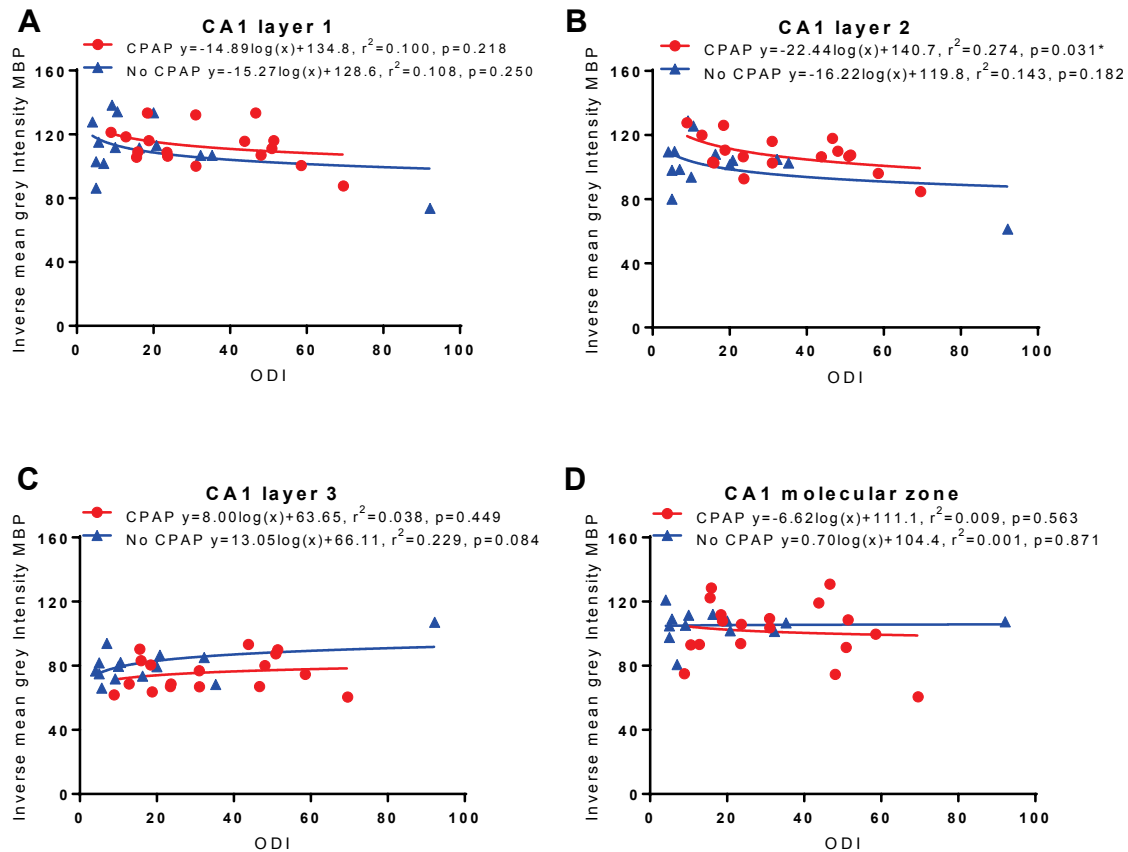


Figure 5.17 The inverse mean grey intensity of MBP staining regressed against OSA severity (ODI), for the CA1 separated by layer (A-D) and by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. * indicates $p < 0.05$.

In the EC, layers 6 showed significant negative correlations between ODI and MBP staining, $r^2 = 0.282$, $p = 0.006$, (Figure 5.18F; Figure 5.14C & D). Layers 1-5 showed no relationship to ODI, indicating that the deep cortical layer (layer 6) of the EC shows decreasing myelin with increasing ODI whereas the superficial layers do not (Figure 5.14C & D). Lighter myelin staining is seen in the deep white matter as well as layer 6 in a patient with a high ODI (Figure 5.14D) compared to a patient with a low ODI (Figure 5.14C). Figure 5.19 shows the same sample separated into CPAP users and non-users. Layers 1-5 show no significant relationships between ODI and myelin staining intensity. Interestingly, layers 1 and 2 show positive relationships for CPAP users and negative relationships for CPAP non-users, possibly indicating that with increasing ODI there is a decrease in myelin for CPAP non-

users and an increase in myelin for CPAP users. However, no statistical significance was found. In layer 6 there were stronger associations between MBP staining intensity and ODI for CPAP users, $r^2=0.315$, $p=0.030$, than for CPAP non-users, $r^2=0.384$, $p=0.073$, compared to the sample as a whole, however only the relationship for CPAP users reached statistical significance. This indicates that CPAP use has little to no effect on the decreased myelin staining in this layer.

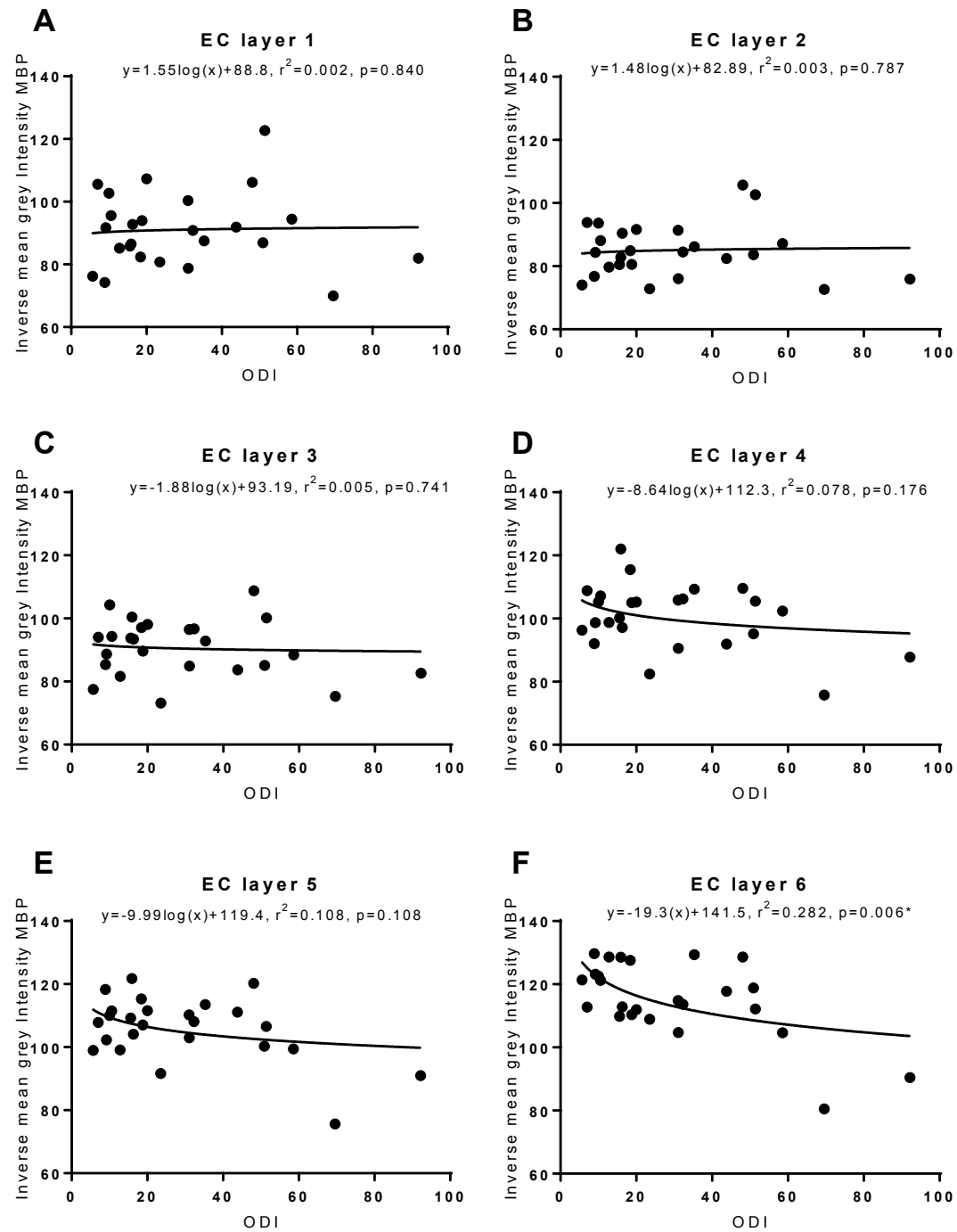


Figure 5.18 The inverse mean grey intensity of MBP staining regressed against OSA severity (ODI), for the EC separated by layer (A-F). EC: entorhinal cortex * indicates $p < 0.05$.

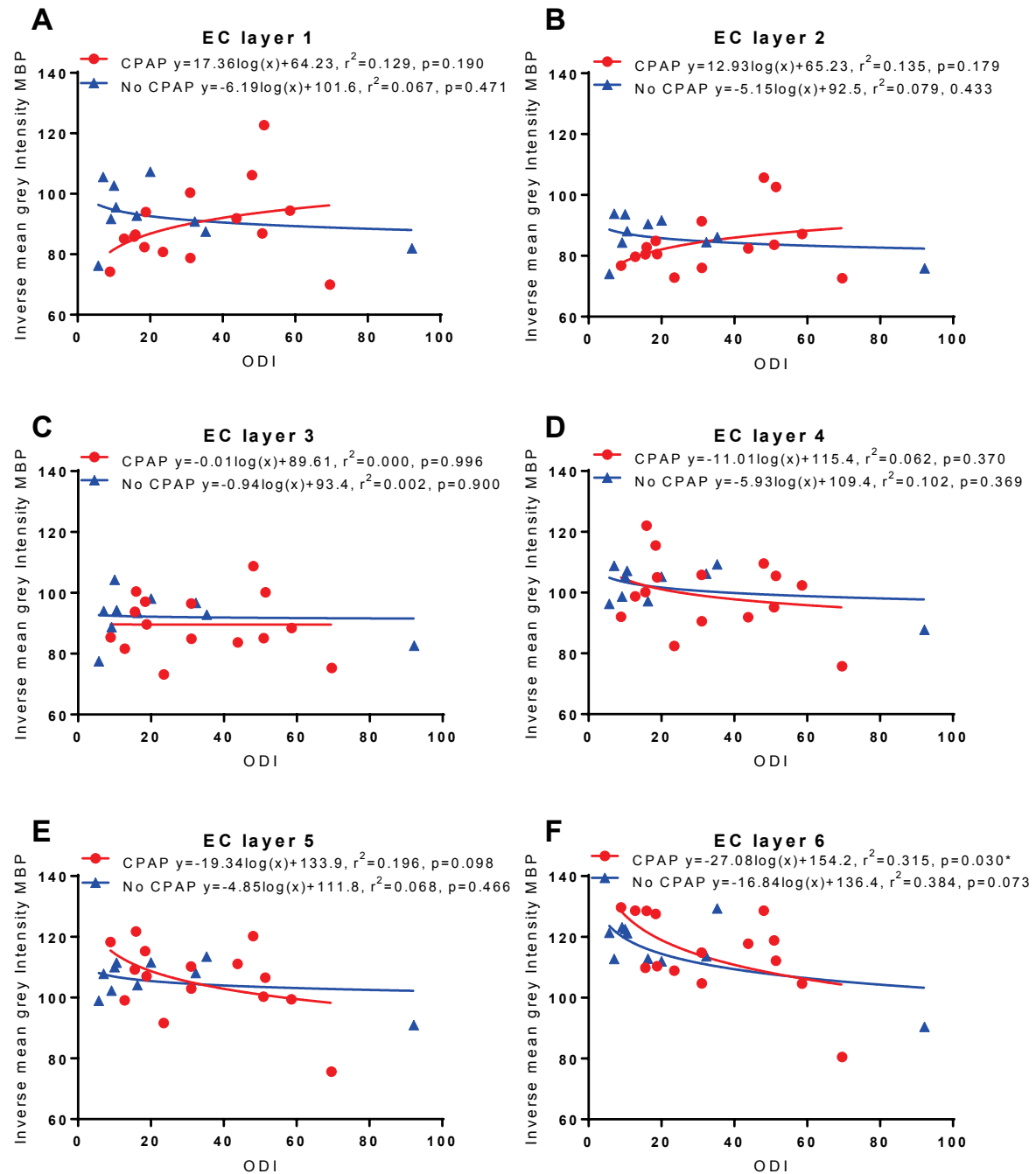


Figure 5.19 The inverse mean grey intensity of MBP staining regressed against OSA severity (ODI), for the EC separated by layer (A-F), separated by CPAP use. CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. EC: entorhinal cortex. * indicates $p < 0.05$.

In the deep white matter of the EC, a strong significant negative correlation was seen between ODI and MBP staining, $r^2=0.390$, $p=0.001$ (Figure 5.20A). With increasing OSA severity, less myelin is seen in the deep white matter of the EC. When separated into CPAP users, $r^2=0.383$, $p=0.014$, and non-users, $r^2=0.609$, $p=0.008$ (Figure 5.20B) the relationship was slightly weaker compared to the whole group but still significant in CPAP users and stronger and significant in CPAP non-users. This indicates that CPAP users and non-users have a similar decrease in MBP staining intensity in the deep white matter. Table 5.3 summarises the significant findings described in the preceding section.

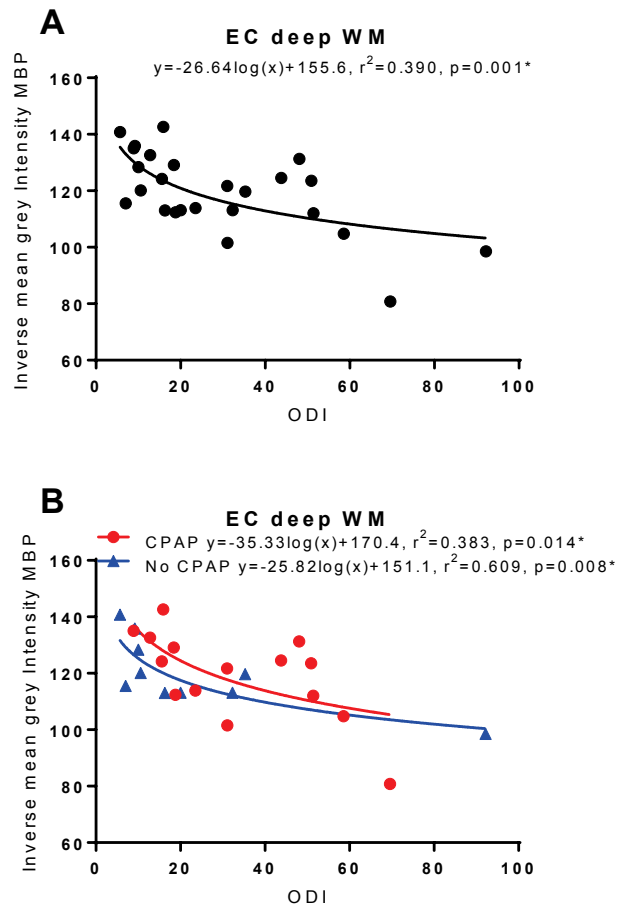


Figure 5.20 The inverse mean grey intensity of MBP staining regressed against OSA severity (ODI), for the deep white matter of the EC, as the complete sample (A) and separated by CPAP use (B). CPAP users are indicated by red circles, CPAP non-users are indicated by blue triangles. EC: entorhinal cortex; WM: white matter. * indicates $p < 0.05$.

Table 5.3 Summary table of significant changes in myelin staining intensity

	Direction and magnitude of change as a function of increasing ODI	Effect of CPAP use (size of r^2)	Effect of not using CPAP (size of r^2)
CA1 layer 2	none	↓	↓
EC layer 6 MBP	↓	↓	↓
EC deep WM MBP	↓	↓	↓

↓ = smaller reduction, ↓ = moderate reduction, ↓ = larger reduction

5.3.6 Age and hippocampal size/demyelination

It is possible that the significant relationships described in the preceding sections were due to age-related changes, rather than to the effects of OSA severity. To investigate this possibility, each of the significant correlations described above were also correlated against patient age. The relationships between measures of hippocampal size and patient age are shown in Figures 5.21 and 5.22. A significant relationship was found between age and the cortical thickness of layer 2 of the CA1, $r^2=0.258$, $p=0.003$. However, none of the other layers of the CA1, the EC or the dentate gyrus measures were significantly correlated with age, indicating that patient age cannot account for the correlations described in the preceding sections.

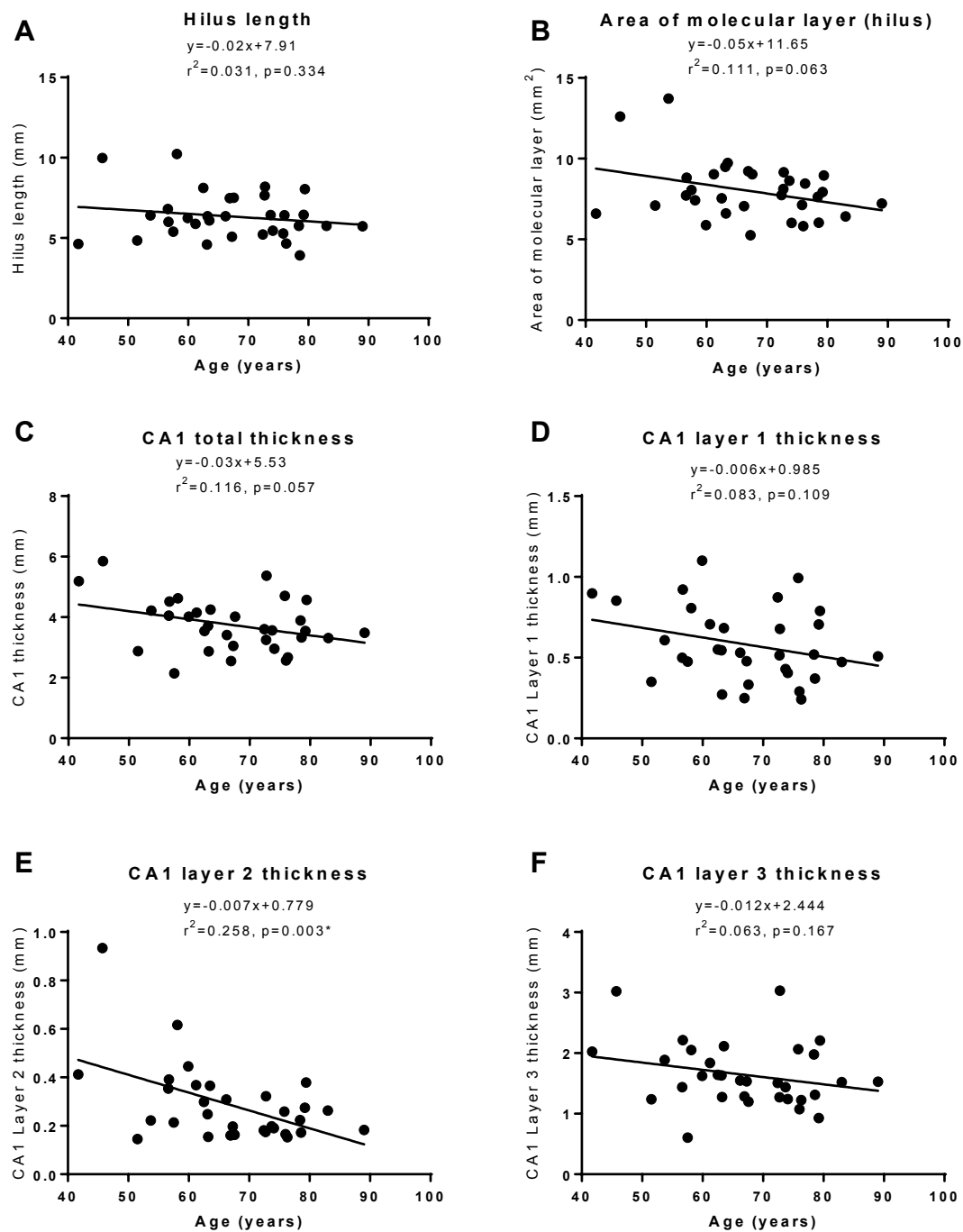


Figure 5.21 Patient age regressed against hilus length (A), area of the molecular layer of the dentate (B), total cortical thickness of the CA1 (C), thickness of layer 1 of the CA1 (D), thickness of layer 2 of the CA1 (E) and thickness of layer 3 of the CA1 (F). * indicates $p < 0.05$.

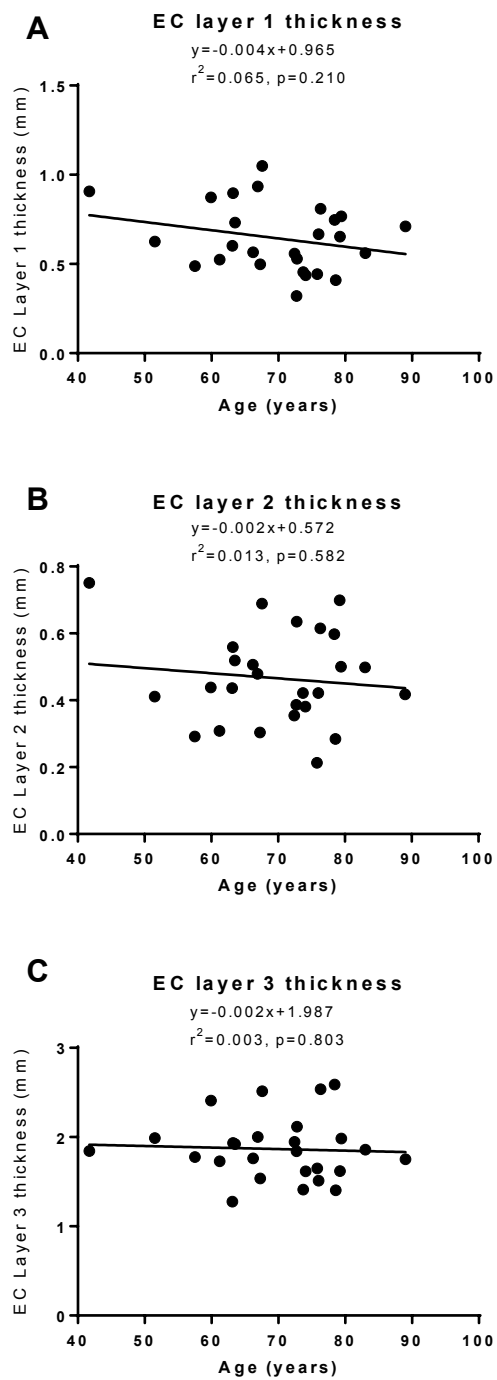


Figure 5.22 Patient age regressed against layer thickness in the EC for layers 1 (A), 2 (B) and 3 (C). EC; entorhinal cortex.

Figure 5.23 shows the relationships between MBP staining intensity and patient age. There are no significant relationships between MBP staining intensity and age, indicating the patient age cannot account for the correlations described in the preceding sections.

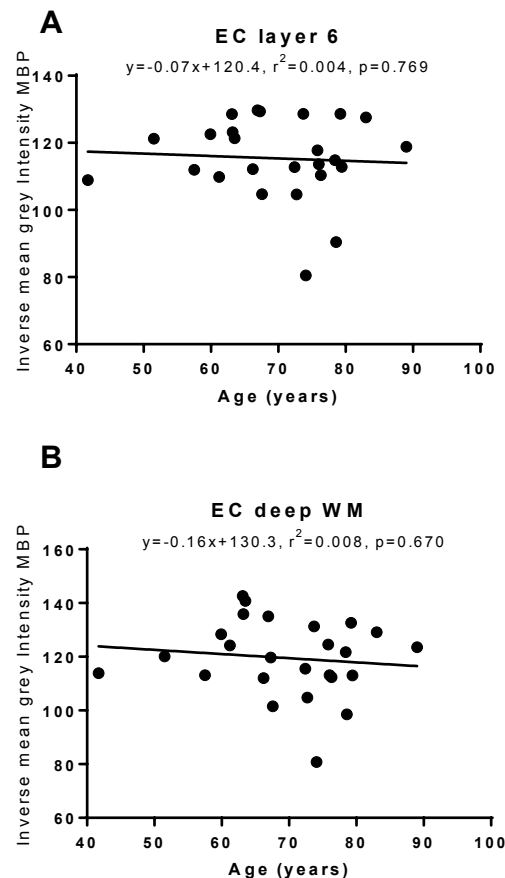


Figure 5.23 Patient age regressed against MBP staining intensity in the EC for layer 6 (A) and the deep white matter (B). EC: entorhinal cortex; WM: white matter.

5.4 Discussion

OSA is thought to be associated with cortical atrophy and demyelination. However, this has only been investigated using animal models and MRI. Histopathology was used in the present study to directly investigate changes in cortical thickness and myelin in OSA brains. The

current study has found that individual layer variations in cortical thickness and myelin staining intensity in the hippocampus are correlated with the severity of OSA.

There is hippocampal loss associated with increasing severity of OSA. In the dentate gyrus, we found that with increasing ODI the length of the hilus and the area of the molecular layer decreased. In the CA1, as ODI increased the overall cortical thickness decreased, as well as the thickness of specific layers of the CA1: layers 1, 2 and 3. In the entorhinal cortex, increasing OSA severity was associated with decreased thickness of layer 1 and a trend towards decreased thickness in layer 3. No neuronal loss was seen in any of the four CA regions examined, therefore decreased cortical thickness in the CA1 and the dentate gyrus are not due to neuronal loss. Neuronal loss may account for the reduced cortical thickness of layer 1 and 3 in the EC. Neuronal atrophy may also be occurring in these regions leading to decreased cortical thickness with no change in neuronal numbers. Another possibility is loss of axons. However, this is unlikely to account for the decreased area of the molecular layer of the dentate, CA1 or the cortical thickness in the EC as no reduction in myelin staining intensity was seen in these regions. Decreased myelin staining was seen in layer 6 of the EC and the deep white matter without simultaneously decreased layer thickness.

The present study found that the extent of cortical atrophy is more severe in the CA1 region compared to the EC. Interestingly, the CA1 has been found to be the most vulnerable region of the hippocampus to hypoxic injury (Duvernoy et al., 2013; Wilde, Pringle, Wright, & Iannotti, 1997). The EC may be protected by the presence of reactive astrocytes. Aviles-Reyes and colleagues suggest that the involvement of reactive astrocytes, including the upregulation of GFAP, accounts for the lack of neuronal death seen in animals that are exposed to IH (Aviles-Reyes et al., 2010). Indeed, an increase in the number of intensely labelled GFAP-positive astrocytes was found in the EC in OSA brains (see Chapter 4) whereas no evidence of reactive astrocytosis was seen in the CA1.

Cortical thinning and myelin loss in the present study were found in regions involved in the polysynaptic and direct pathways of memory processing. Figure 5.25 shows these pathways and the regions found to be affected by OSA in the present study. The molecular cell layer of the dentate and layer 3 of the CA1 are locations of synaptic connections in the polysynaptic pathway; both had decreasing cortical thickness with increasing severity of OSA. Further, we

saw decreased myelin in the entorhinal cortex (layer 6 and deep white matter); a major output of the direct and polysynaptic pathways. The entorhinal cortex fibres of the direct pathway connect to multiple cortical regions, mostly the association cortices (Canto, Wouterlood, & Witter, 2008; Duvernoy et al., 2013). CA1 layer 3 pyramidal neurons receive synapses from both the polysynaptic and direct memory pathways. The similarity between the locations of synaptic connections and the regions of cortical thinning suggest damage to both memory pathways, with implications for episodic, semantic and spatial memory in OSA patients. Indeed previous research has found impairment in all three types of memory in OSA patients (Daurat, Foret, Bret-Dibat, Fureix, & Tiberge, 2008; Twigg et al., 2010; Wallace & Bucks, 2012), although one study found more severe impairment in episodic memory than semantic memory (Twigg et al., 2010).

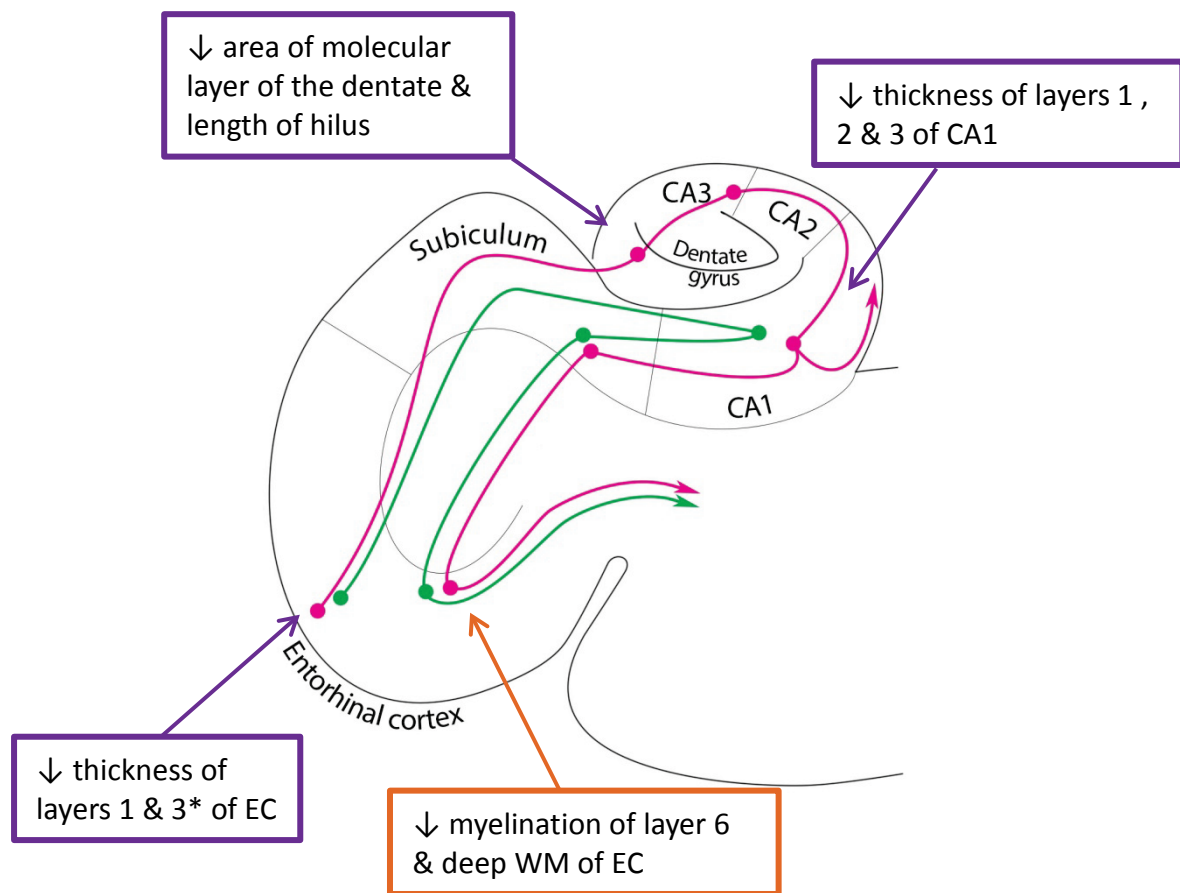


Figure 5.24 Diagram of the polysynaptic (pink) and direct (green) memory pathways in the hippocampus and summarised findings from the present study showing regions of decreased cortical thickness (purple boxes) and decreased myelin staining (orange boxes). Notice the overlap between synaptic connections of the memory pathways and regions of cortical thinning and demyelination. Based on circuitry described by Duvernoy et al. (2013). * Trend for a relationship

For cortical thickness measures, there was a general strengthening of the relationship with ODI among CPAP non-users, and no change or a weakening of the relationship with ODI for CPAP users. This pattern was evident in the dentate gyrus, CA1 and EC, even though some layers of the EC showed no significant relationship for the whole sample (Table 5.2). This finding suggests that more substantial atrophy occurs in CPAP non-users, and that users of

CPAP may be protected against cortical atrophy. This finding is of particular interest for the regions associated with the direct and polysynaptic memory pathways, as it implies that CPAP may be able to protect against hippocampal atrophy and memory impairment. This conclusion is supported by MRI studies which have found that OSA patients with decreased hippocampal volume experience a subsequent increase in hippocampal volume after as little as three months of CPAP treatment (Canessa et al., 2011; H. Kim et al., 2016). Further, Canessa et al. (2011) found parallel improvements in short-term and long-term memory after three months of treatment.

Conversely, CPAP use is not consistently protective against myelin loss. Compared to the whole sample, CPAP non-users had stronger relationships between ODI and myelin staining in the EC layer 6 and the deep white matter (Table 5.3). However, CPAP users also had a stronger relationship in the EC layer 6, while a slightly weaker but still significant relationship was seen in the deep white matter. Additionally, CPAP users had a stronger and significant relationship in the CA1 layer 2 despite no relationship for the whole sample. This inconsistency suggests that CPAP is less effective at protecting against myelin loss. Previous MRI research has found three months of CPAP treatment provided little improvement in white matter integrity for OSA patients, whereas more significant improvements in white matter integrity were seen after 12 months of treatment (Castronovo et al., 2014). The duration of CPAP treatment use in the current study is unknown; long-term CPAP use or early intervention may be required to protect against white matter damage.

The patterns of cortical atrophy and demyelination seen in the present study are similar to changes seen in the hippocampus in MCI and AD. Thinning of the CA1 and white matter damage are commonly seen in the AD brain (Hyman, Van Hoesen, Kromer, & Damasio, 1986; Kerchner et al., 2010; Salat et al., 2010). Evidence suggests that white matter deteriorates early in the pathological process of AD (Bartzokis, 2004; X. Zhan et al., 2014). Indeed, damage to white matter is present in the brains of patients with MCI, who have no discernible neuronal loss (Agosta et al., 2011). Considering that A β plaques and NFT accumulation has been demonstrated in OSA brains (see Chapter 3) it is not surprising that decreased myelin is evident in the same region. Neuronal loss is evident in AD in the CA1, CA2, CA3, CA4 and layer 2 of the entorhinal cortex (Delli Pizzi et al., 2016; Gómez-Isla et al., 1996; Wisse et al., 2014), conversely no neuronal loss was found in the present study.

However, preclinical AD patients who had A β plaques and/or NFTs but no clinical symptoms, had no neuronal loss in the CA1 or layer 2 of the entorhinal cortex (Price et al., 2001); which is similar to the pattern seen in the present study. In cognitively normal elderly people, atrophy of the CA1 predicts those who will develop MCI and then AD at follow up (Apostolova et al., 2010). This trend has implications for OSA, as CA1 atrophy may be a useful biomarker for predicting those OSA patients who are at increased risk of MCI/AD. Improvements in the spatial resolution of MRI may enable this parameter to be used in living patients. Additionally, CPAP treatment could be targeted to these individuals to potentially delay or prevent further neurodegeneration.

There are limitations of the present study that could be improved in future studies, including a larger sample size and more detailed information regarding the length and compliance of CPAP use. An additional limitation of the present study is that the scarcity of tissue restricted the number of hippocampal sections that could be obtained and consequently prevented a complete stereological cell count estimate, which is often performed in animal models to obtain estimates of neuronal number. Consequently, the counts are actually estimates of the packing density of neurons rather than the total number of neurons. Despite these limitations, the present study has, for the first time, shown that cortical thinning and demyelination occur in specific regions of the hippocampus and entorhinal cortex of OSA patients, and that CPAP use may be protective against cortical thinning but not demyelination.

Chapter 6 Discussion & Conclusions

6.1 Introduction

The main aim of the present thesis was to investigate what neuropathological changes occur in the hippocampus and LC of OSA patients, and then to determine the extent to which such changes resemble those seen in AD, and whether they are related to the severity of OSA. Further, the association with CPAP treatment was also investigated, as was age as a possible contributing factor. These investigations revealed that the extent of neuropathological changes increases with increasing severity of OSA. Specifically, increased OSA severity was related to increased numbers of NFTs and A β plaques, increased signs of reactive gliosis, decreased thickness of cell layers and decreased amounts of myelin. Similar neuropathological changes are observed in AD, where they appear to begin in the hippocampus and overlying cortex.

It was also found that the relationship between OSA severity and neuropathology is stronger in CPAP non-users and weaker in CPAP users. This was found for the relationships with NFTs, A β plaques, most markers of gliosis and some decreases in cell layer thickness. The relationship between OSA severity and demyelination did not appear to be influenced by CPAP use. These findings indicate that CPAP has some capacity to protect the brain from neuropathological damage, and suggest that not all aspects can be improved with treatment. Age was found to be associated with OSA severity and it is likely to be a factor in some of the relationships found. The relationships between OSA severity and NFTs, some measures of reactive gliosis and cortical thickness of the CA1 all have an age component to them. The implications of these findings will be discussed in detail below, as will the limitations of the project and suggestions for future research.

Figures 6.1- 6.4 are schematic summary representations of the main findings of the present thesis, including the relationship between different neuropathological features and OSA severity (low and high OSA) and between CPAP users and CPAP non-users.

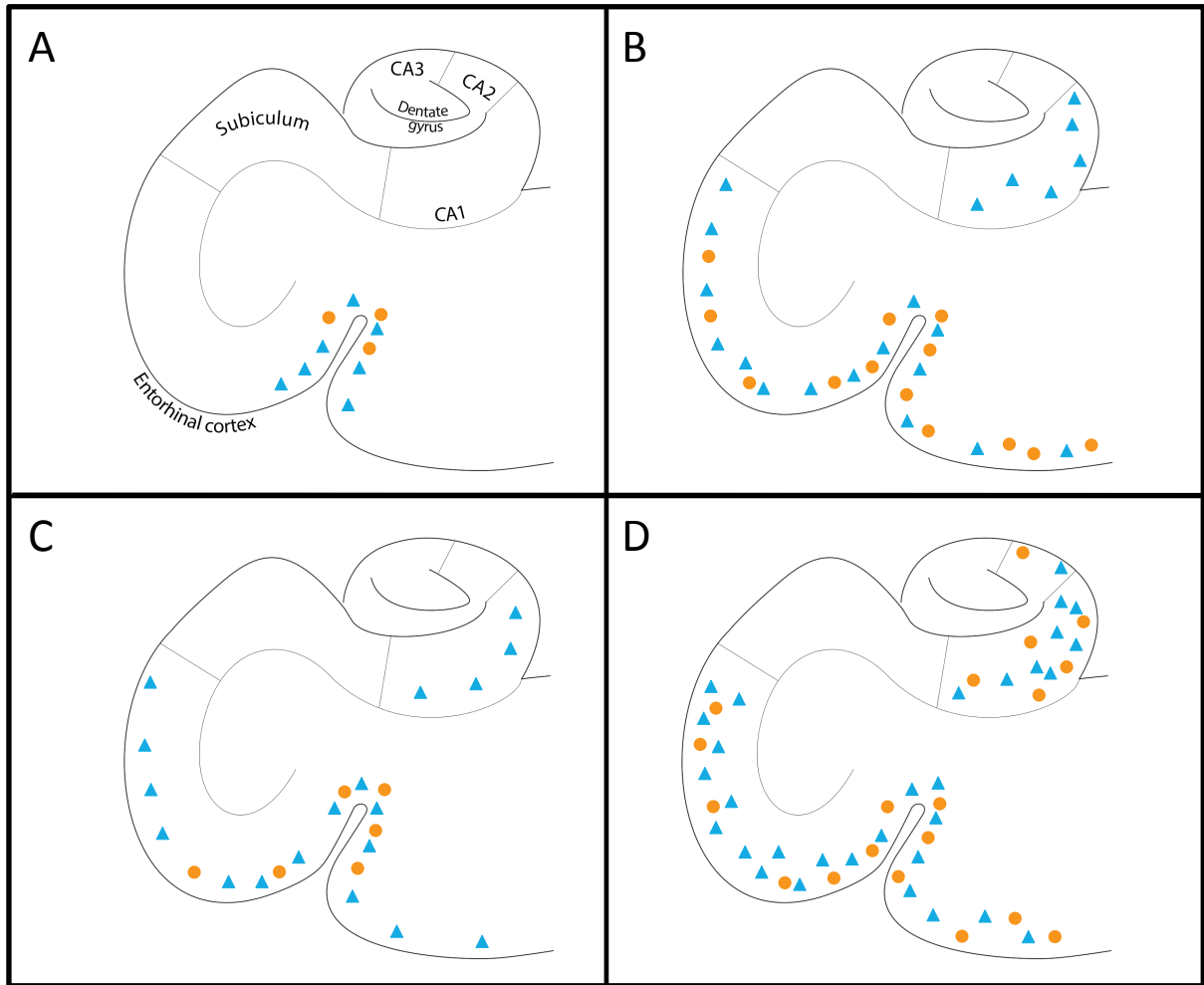


Figure 6.1 Schematic illustrations summarising the difference in the distributions of A β plaques and NFTs in the hippocampus in low (A) and high (B) OSA, and in CPAP users (C) and CPAP non-users (D). Blue triangles represent NFTs and orange circles represent A β plaques. Notice the increased burden of NFTs and A β plaques in the high OSA condition compared to the low OSA condition. Also note the decreased numbers of NFTs and A β plaques in the CPAP users compared to CPAP non-users.

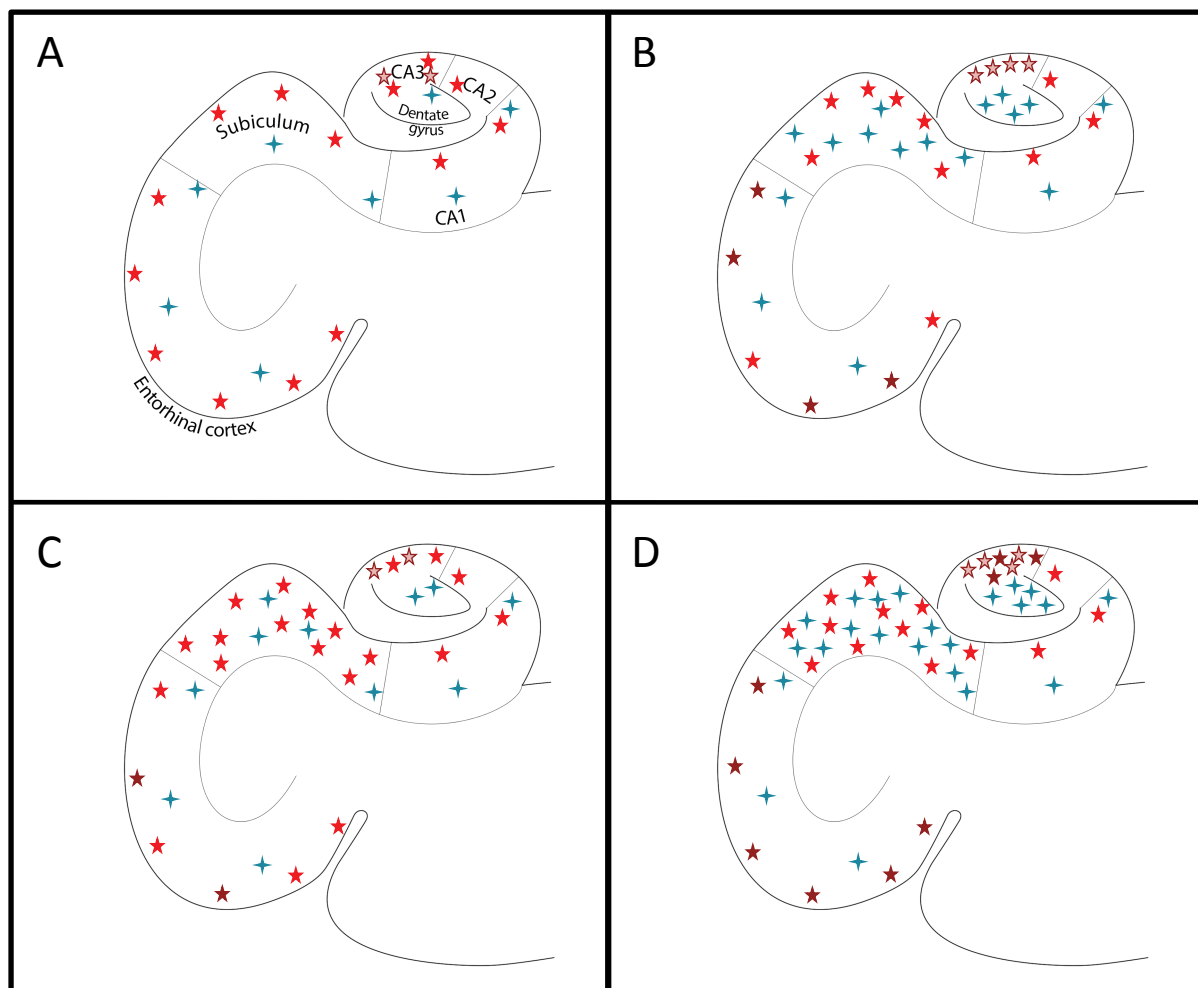


Figure 6.2 Schematic illustrations summarising the difference in the distributions of reactive gliosis in the hippocampus in low (A) and high (B) OSA, and in CPAP users (C) and CPAP non-users (D). Red stars represent astrocytes, dark red stars represent astrocytes with increased staining intensity, pale red stars represent astrocytes with decreased staining intensity and teal stars represent microglia. Notice the increase in the number of microglia in the subiculum and hilus, the increase in astrocytes in the subiculum, the increase in intensely-stained astrocytes in the entorhinal cortex and the decrease in intensely stained astrocytes in the CA3 in the high OSA condition compared to the low OSA condition. Also note the decreased extent of reactive gliosis in CPAP users compared to CPAP non-users.

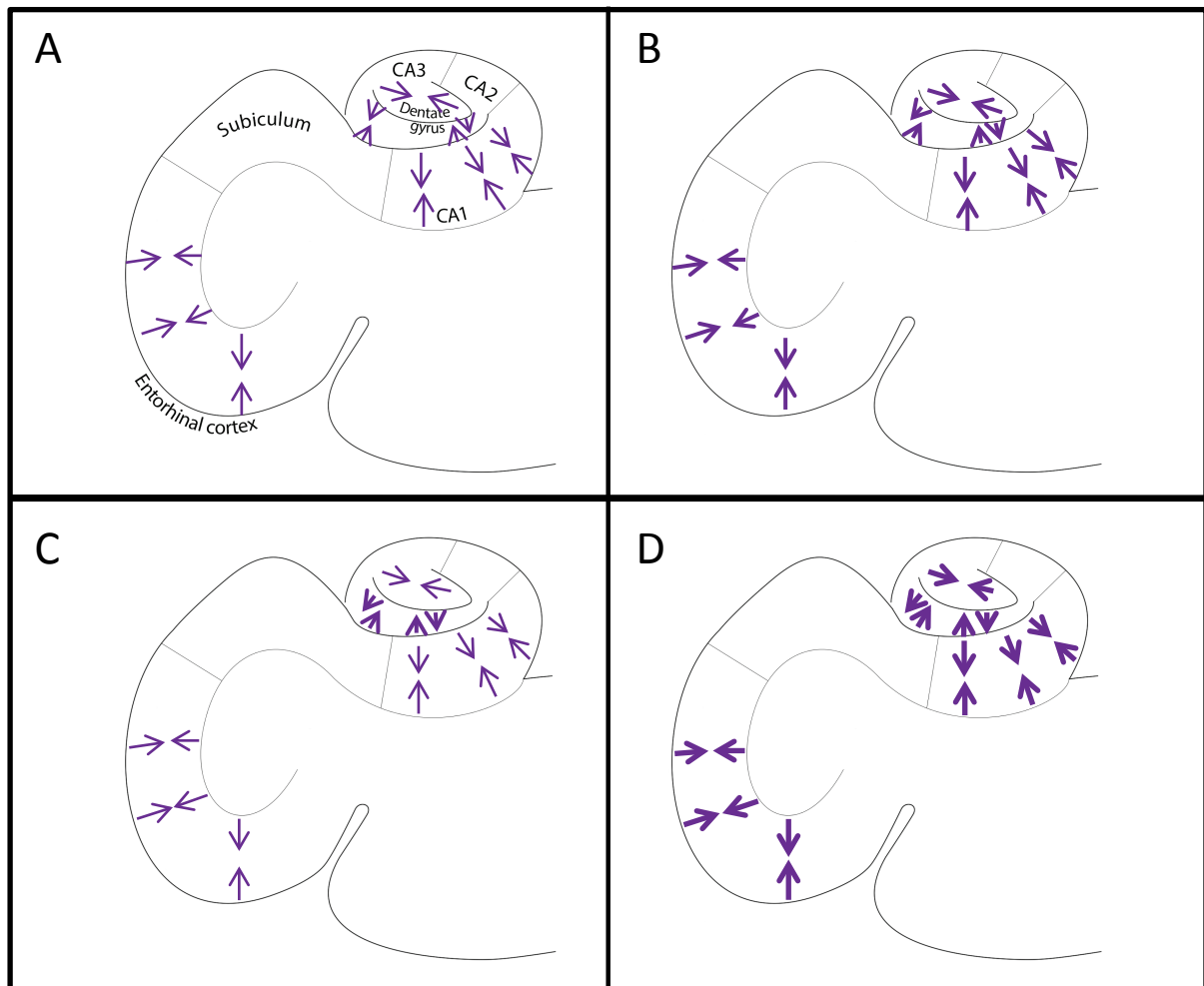


Figure 6.3 Schematic illustrations summarising the difference in the thickness of hippocampal cell layers in low (A) and high (B) OSA, and in CPAP users (C) and CPAP non-users (D). Purple arrows represent the region of cortical thinning, thicker arrows indicate more atrophy. Notice the greater atrophy in the high OSA condition compared to low OSA condition. Also note that there is less atrophy in CPAP users compared to CPAP non-users.

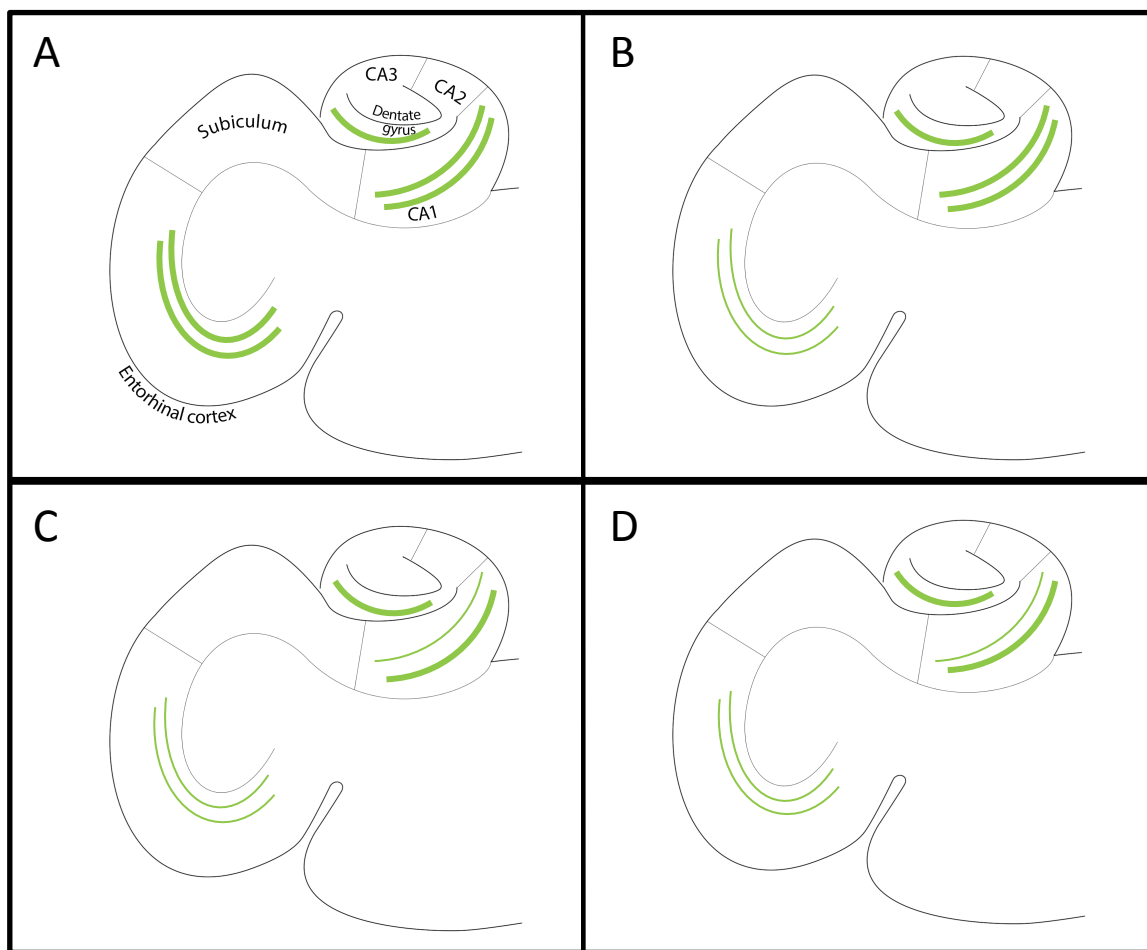


Figure 6.4 Schematic illustrations summarising the difference in the degree of demyelination in the hippocampus in low (A) and high (B) OSA, and in CPAP users (C) and CPAP non-users (D). Green lines represent myelin, thinner lines indicate less myelin. Notice there is less myelin in the entorhinal cortex of the high OSA condition compared to the low OSA condition. Also note the similarity between the amount of myelin in CPAP users and CPAP non-users.

6.2 A β plaques and NFTs in OSA

AD is characterised neuropathologically by the accumulation of A β plaques and NFTs, particularly in the hippocampus and surrounding cortex. Significant amounts of tissue

atrophy, gliosis and demyelination accompany these plaques and tangles (Miller et al., 1980; Rodriguez Arellano, 2011; Sjöbeck et al., 2005). Perhaps the most striking finding to emerge from the present study is that the neuropathological changes observed in severe OSA closely resemble those seen in the early stages of AD. This similarity raises the interesting possibility that the neuropathological changes seen in both disorders might have a common origin. In other words, since OSA generally begins in midlife, decades before the onset of AD, perhaps hypoxia, sleep fragmentation or a combination of the two increases the vulnerability of the brain to whatever agent causes AD.

A β plaques and NFTs were found in the brainstems of OSA patients; however their presence was not related to OSA severity or to the presence of hippocampal pathology. A β and tau pathology are also observed in the brainstems of AD patients, yet their relationship to hippocampal pathology has not been resolved. One hypothesis posits that NFTs and phosphorylated tau accumulate in the brainstem prior to their appearance in hippocampal regions (Braak & Del Tredici, 2015; Simic et al., 2009). Models of axonal transportation of misfolded tau protein have been postulated to explain this (Grinberg et al., 2009; Simic et al., 2009). Indeed, there are axonal pathways between the specific brainstem nuclei that accumulate phosphorylated tau and the affected regions of the basal forebrain and hippocampus. For instance, the LC has noradrenergic projections to the hippocampus and amygdala, while the dorsal raphe nucleus has serotonergic projections to the basal forebrain. Despite these compelling links, the present study found no association between the presence of brainstem neuropathology and the presence of hippocampal pathology. Hippocampal pathology was generally far more extensive than that observed in the brainstem, and in many cases few NFTs and no A β plaques were observed in the brainstem. A lack of an association has also been reported by some neuropathological investigations of AD (Haglund, Sjöbeck, & Englund, 2006). Since the present findings show that hippocampal pathology is not dependent on the presence of brainstem pathology, they do not support the hypothesis that NFTs are transported from the brainstem to the hippocampus. In OSA it appears that the brainstem pathology occurs after, or concurrently with, the appearance of hippocampal pathology. Indeed, these results are more consistent with the notion that the axonal transport of tau occurs in a retrograde direction, from the hippocampus to the LC. However, it is

equally likely that the NFTs accumulate independently at both sites and are the consequence of hypoxic injury, with the brainstem being less sensitive to hypoxia.

In the present study, the burden of A β plaques was found to increase in the hippocampus as OSA severity increased (Figure 6.1A & B). It has been suggested that both hypoxia and sleep fragmentation can increase the burden of A β in the brain via separate mechanisms. Hypoxia increases the expression of HIF-1 α (Chaves et al., 2010), which in turn increases BACE1 expression, consequently increasing the production of A β (Guglielmotto et al., 2009; X. Sun et al., 2006). Curiously, HIF-1 α has been suggested to play a role in the pathogenesis of AD and MCI (Iyalomhe et al., 2017). Additionally, sleep disruption leads to decreased clearance of A β from the brain (L. Xie et al., 2013), suggesting a role for the sleep fragmentation that occurs in OSA. While these two mechanisms can explain the general increase in A β levels as OSA severity increases, they cannot account for the specific spatiotemporal pattern of A β accumulation observed. A β plaques were most commonly seen in the transentorhinal region, around the collateral sulcus, which is the same location that A β plaques begin to accumulate in AD. Interestingly, the CA1 is the most vulnerable region of the brain to hypoxia (Duvernoy et al., 2013), yet this region is not the first part of the hippocampus to be affected by A β plaque accumulation in OSA. Since hypoxia and sleep fragmentation cannot explain why A β deposition begins at the collateral sulcus and then spreads towards the hilus, an additional factor(s) must be involved. At this stage, the factor(s) remains unknown, however it could be the same factor(s) that precipitates A β deposition in AD, which also begins at the collateral sulcus and then spreads towards the hilus (Braak & Braak, 1991; Thal et al., 2000).

6.3 Gliosis and hippocampal atrophy in OSA

Reactive gliosis, hippocampal atrophy and neuronal loss are all observed in AD (Kril et al., 2004; Kril et al., 2002; Verkhatsky et al., 2010). These neurodegenerative changes have also been speculated to occur in OSA. Studies conducted on animal models of IH have reported reactive gliosis and some evidence of neuronal loss when compared to control animals (Aviles-Reyes et al., 2010; D. Gozal, Row, Kheirandish, et al., 2003; Smith et al., 2013). Additionally, MRI studies of OSA patients have detected reductions in hippocampal volume compared to controls (Canessa et al., 2011; Gale & Hopkins, 2004; Macey et al., 2002), and

MRS studies of OSA patients have found changes in metabolites that are considered to represent glial injury in the temporal lobe (Sarchielli et al., 2008; Sharma et al., 2010; Yadav et al., 2014). While decreased levels of N-acetylaspartate, a marker of neuronal injury, have been observed in the brains of OSA patients, these changes were found in the frontal cortex and cerebral white matter, not in the hippocampus (Alchanatis et al., 2004; Kamba et al., 1997; O'Donoghue et al., 2012). In AD by contrast, the levels of N-acetylaspartate are significantly reduced in the hippocampus, where substantial neuronal loss is also seen (Graff-Radford & Kantarci, 2013; N. Zhang et al., 2014).

The present project found evidence of gliosis and hippocampal atrophy in OSA (Figure 6.2A & B; Figure 6.3A & B). The CA1 is the hippocampal region most vulnerable to hypoxia (Duvernoy et al., 2013; Kreisman, Soliman, & Gozal, 2000), so it may be significant that the CA1 had the most extensive atrophy and was the only region where the number of NFTs was correlated with ODI. These degenerative changes corroborate the MRI studies that have reported volume reductions in the hippocampus in severe OSA. Despite the reduced thickness of the cell layers observed in the CA1, we detected no evidence of reactive gliosis or reduced numbers of neurons. Taken together, these findings may indicate that neurons in the CA1 shrink in response to intermittent hypoxia, but they do not die. Perhaps in this state the neurons in CA1 no longer release cytokines in response to hypoxia, so no stimulus for gliosis remains. As discussed in Chapter 4, this situation could occur if the neurons in CA1 become adapted to the repeated episodes of hypoxia.

While it is possible that OSA causes the shrinkage of neurons rather their death, it is also possible that the methodology employed in the present study was not sensitive enough to detect neuronal loss. Ideally, stereological cell counting from multiple serial sections of the hippocampus would have been conducted in order to estimate the total number of neurons present in the whole hippocampus. This was not possible in the present study, due to the extremely limited amount of tissue available. The neuronal cell counts in the present study were based on one section per brain; therefore the counts actually estimate the packing density of neurons in the hippocampus, not the total number. This method would not detect cell loss if the volume of the hippocampus shrinks while the neuronal packing density remains unchanged. We will have to wait until immunohistochemical studies with access to tissue from the entire hippocampus of OSA patients are able to resolve this issue.

The present study found that A β plaques and NFTs accumulate in the entorhinal cortex in response to OSA; additionally an increase in the intensity of GFAP-immunostaining of astrocytes was found in the same location. In AD, reactive astrocytes are commonly found surrounding A β plaques, these astrocytes frequently have increased expression of GFAP and S100b and decreased GS expression (Robinson, 2000), however this pattern was not seen in the present study. High OSA was associated with an increase in the number of intensely labelled GFAP-immunoreactive astrocytes in the entorhinal cortex and CA3 (CPAP non-users only) of the hippocampus, a decrease in the number of intensely labelled S100b-immunoreactive astrocytes in the CA3, and no changes in GS expression were observed at any location. This suggests that hypoxia and sleep fragmentation from OSA initiate reactive gliosis differentially to that seen in AD. Indeed, Zamanian et al. (2012) found that at least 50% of the changes in gene expression of reactive astrocytes were specific to the type of brain injury induced. Thus, the present results are not surprising given the heterogeneous response of astrocytes to injury (Anderson et al., 2014; Zamanian et al., 2012).

The present study found a significant amount of demyelination in relation to OSA severity (Figure 6.4A & B). In the entorhinal cortex, demyelination was found in layer 6 and the deep white matter suggesting a loss of myelin or axons in response to OSA severity. This finding is consistent with reports from imaging studies of OSA patients that have found significant reductions in white matter integrity in the hippocampus (H. L. Chen et al., 2015; Kumar et al., 2014). Both studies concluded that myelin loss, rather than axonal loss, underpinned the reduction in white matter volume.

In AD, hippocampal atrophy appears to be due to both neuronal loss (West et al., 1994), and to white matter deterioration and myelin loss (Alves et al., 2012; Sjöbeck et al., 2005).

Studies suggest that white matter loss may occur early in the pathological process (Bartzokis, 2004), and perhaps may even trigger AD pathogenesis (Bartzokis, 2011). The results of the present study found demyelination, cortical atrophy and accumulation of A β plaques and NFTs all in the entorhinal cortex, the first location to show neuropathology in AD. Imaging studies in AD have found that decreased white matter volume correlates with grey matter volume loss in the hippocampus (Agosta et al., 2011). However, the present study found significant atrophy in CA1 with no concurrent white matter loss. Significant demyelination and atrophy were found in the entorhinal cortex, but were seen in different cortical layer

suggesting that grey matter and white matter loss are not correlated in OSA. While we were unable to find evidence of neuronal loss or axonal loss, our methodology would have prevented the detection of such loss unless it was very extensive. Although the white and grey matter changes seen in OSA are not as severe or extensive as those seen in mid- or late-stage AD, it needs to be borne in mind that the age of subjects in the present study ranged from 41.7 to 83 years with a mean of 67 years. By contrast, AD does not become common until after age 75. Thus the appearance of AD-like atrophy and demyelination in relatively young OSA patients supports the notion that OSA may predispose individuals to develop AD in later life.

6.4 The influence of CPAP use on neuropathology

The present study found that many of the relationships between OSA severity and neuropathology differed according to CPAP use. CPAP non-users often had stronger relationships between ODI and neuropathology, whereas CPAP users had weaker, sometimes non-existent, relationships. CPAP use protected against NFT and A β plaque accumulation, dentate gyrus and CA1 atrophy, some entorhinal cortex atrophy and most of the glial relationships (Figure 6.1C & D; Figure 6.2C & D; Figure 6.3C & D). These findings indicate that some of the neuropathological damage can be lessened by CPAP treatment, and this neuroprotective effect may underpin the cognitive improvements seen in patients who are treated with CPAP (Ferini-Strambi, Marelli, Galbiati, & Castronovo, 2013).

CPAP is effective at restoring sleep architecture to a normal pattern and consistently reduces daytime sleepiness in OSA patients (Kushida et al., 2012; Sullivan et al., 1981). Further, increased hippocampal volume has been found following CPAP treatment of patients with decreased baseline hippocampal volume (Canessa et al., 2011; H. Kim et al., 2016). This result is consistent with findings from the present project of weaker, non-significant relationships between OSA severity and hippocampal atrophy in patients using CPAP. Additionally, the present study was able to identify specific regions of the hippocampus that benefit from CPAP treatment including the CA1, some layers of the EC and the CA4 hilus length. It is interesting that the CA1 is the most vulnerable region to hypoxia, it experiences the most severe atrophy (overall thickness and layers 1, 2 and 3 all showed thinning) in OSA

patients, yet it also shows the most improvement from CPAP use (all layers with cortical thinning had significantly less atrophy in CPAP users). This suggests that while the CA1 is vulnerable in OSA, it is not beyond repair with diligent CPAP use.

CPAP users also had lower burdens of NFTs and A β and less neuroinflammation in the hippocampus. This observation suggests that while OSA may accelerate the pathogenesis of AD-like pathology in the brain, many of these changes can be prevented with CPAP treatment. This has implications for the treatment of similar pathology in other conditions, including AD, where the use of CPAP treatment for three weeks has been shown to improve cognition in patients with comorbid OSA (Ancoli-Israel et al., 2008). Another study found that CPAP was able to slow the cognitive decline of patients with AD and OSA over a mean follow-up period of 4 years (Troussière et al., 2014). These studies suggest that AD symptoms can only be slowed and not halted or reversed with CPAP treatment, however the patients would be expected to have quite severe neuropathology and perhaps earlier intervention could provide more substantial benefits. This possibility could be investigated by treating patients with less severe cognitive deficits such as MCI patients or people with increased risk of developing AD, to determine if the rate of conversion to AD can be reduced with CPAP treatment.

The cross-sectional design of the present study did not allow us to differentiate between the possibilities that CPAP slows or reverses neuropathological damage. It is not known whether the CPAP-treated patients had a greater burden of pathology before they commenced CPAP, which subsequently decreased as a result of treatment, or whether CPAP slowed the accumulation of additional pathology. Either way, the present cross-sectional study has demonstrated that a medical intervention is capable of reducing the burden of AD-like neuropathology over time, when compared with the burden in untreated individuals. As far as we are aware, the extent of this protective effect is far greater than observed for any other medical intervention tested thus far, and it raises the real possibility that CPAP could be used to slow the progression of AD-like neuropathological changes in OSA patients, at least during the prodromal stages of AD. A longitudinal study using A β imaging in OSA patients before and after long-term CPAP treatment would provide valuable insights into this possibility, particularly if cognitive testing is conducted as part of the study.

Interestingly, the present study found that CPAP did not have a beneficial effect on demyelination. CPAP users and non-users tended to have very similar relationships for all myelin measures, suggesting that CPAP affords little protection against demyelination (Figure 6.4C & D). By contrast, a study that investigated white matter integrity in OSA patients before and after CPAP treatment found improvements after treatment in the superior longitudinal fasciculus and the uncinate fasciculus (Castronovo et al., 2014). However, as that study did not investigate the hippocampus, it does not contradict the present findings that the beneficial effects of CPAP do not extend to the white matter of the entorhinal cortex and hippocampus.

No evidence of increased myelination in CPAP users was found in the present study, suggesting that white matter damage is resistant to CPAP treatment. Multiple sclerosis research suggests that remyelination is sometimes ineffective due to the prevention of oligodendrocyte progenitor cells (OPCs) differentiating into mature myelinating oligodendrocytes (Kuhlmann et al., 2008). OPC differentiation decreases with age (Shields, Gilson, Blakemore, & Franklin, 1999; Sim, Zhao, Penderis, & Franklin, 2002) and the presence of myelin debris from increasing demyelination can prevent OPC differentiation (Kotter, Li, Zhao, & Franklin, 2006). The presence of myelin debris and the age of the participants (mean age 67 years) in the present study may explain why no indication of remyelination was found in OSA patients treated with CPAP.

CPAP is not pleasant to use, and non-adherence rates have been estimated at between 46-83%, where adherence is defined as at least 4 hours of use per night (Weaver & Grunstein, 2008). The present findings may provide OSA patients, particularly severe OSA patients, with a stronger incentive to use and adhere to CPAP treatment. Additionally, it supports the need to improve the comfort of CPAP devices so that compliance rates can be raised. The present study has highlighted the neuropathological damage that can occur in OSA and has emphasised the benefits of CPAP treatment. Once these findings are disseminated it will raise public awareness of the detrimental effects of OSA on the brain and encourage people who might be at increased risk of having OSA to undergo PSG testing.

6.5 Age and OSA neuropathology

Any research investigating neurodegenerative processes must consider age as a potential contributing factor. The present project found a correlation between age and OSA severity as measured by ODI. This result supports previous reports that increasing OSA severity is related to increasing age (Butner et al., 2013; Leitzen, Brietzke, & Lindsay, 2014), possibly due to degenerative airway changes, including decreased muscle tone and respiratory effort (Gislason, 2014; Punjabi, 2008), both of which are associated with increasing age (Eikermann et al., 2007).

The present project found that the burden of NFTs in the hippocampus of OSA patients increased with age, confirming previous reports for elderly populations (Braak et al., 2011; Price et al., 2001; Price et al., 2009). However as OSA severity also increased as a function of age, the precise contributions of age and OSA severity could not be determined in the present study. In future, a larger sample could use factor analysis to estimate the independent contributions of these two factors to NFT burden. It is noteworthy however, that the present study found no effect of age on A β burden, and hence the increase in A β burden as a function of increasing OSA severity is independent of age. A lack of correlation between age and A β plaques has been reported previously (Price et al., 2009).

The present study also found that the extent of atrophy in the CA1, specifically in layer 2, is selectively influenced by age. While age-related atrophy of the CA1 has been previously reported (Bartsch & Wulff, 2015; Pini et al., 2016; Wisse et al., 2014), a study by La Joie and colleagues (2010) detected age-related atrophy of the subiculum, but not of the CA1. It is significant that the present study did not find an effect of age on atrophy of the entorhinal cortex or any other part of the hippocampus. Consequently the reductions in size observed in two measurements of the CA4, two layers of the CA1 and two layers of the EC are all attributable to the effects of increasing OSA severity.

The present study found that the number of microglial cells in the hilus and subiculum and the number of GFAP-positive astrocytes in the subiculum are related to age. Reactive gliosis is a common occurrence in the ageing brain. The numbers of S100b-positive and GFAP-positive astrocytes increases with age (Mrak, Griffin, & Graham, 1997; Sheng et al., 1996; Unger, 1998), as well as the number of reactive microglia in the mesial temporal lobe (Sheng,

Mrak, & Griffin, 1998). Thus increased age may have contributed to our findings of increased numbers of microglia in the hilus and subiculum, and increased numbers of GFAP-positive astrocytes in the subiculum of patients with more severe OSA. However, the present study also found additional evidence for significantly increased gliosis in the CA3 and entorhinal cortex that were not influenced by age, and hence are likely to be directly attributable to OSA severity.

Previous studies have reported that the total number of oligodendrocytes in the brain decreases with age (Fabricius, Jacobsen, & Pakkenberg, 2013; Pelvig et al., 2008). Although oligodendrocytes were not counted in the present study, no age-related decreases in myelin expression were found. MRI studies have frequently reported decreases in white matter volume with increasing age (Bartzokis, 2004; Bartzokis et al., 2001; Madden et al., 2012; Marner, Nyengaard, Tang, & Pakkenberg, 2003; Meier-Ruge, Ulrich, Bruhlmann, & Meier, 1992), however such studies have generally examined the deep white matter of the cortical lobes, which is susceptible to lesions resulting from vascular hypertension. The present author is not aware of any reports showing an age-related decline in myelination of the human hippocampus.

The present study confirmed previous reports that increasing age is associated with an increased burden of NFTs, increased gliosis and increased atrophy of layer 2 of the CA1. These features also correlated with increasing OSA severity, and the present study was not able to determine the extent to which age influenced the apparent correlation between these measures and OSA severity. However, given that there was no age difference between CPAP-users and non-users, the neuropathology was likely driven by OSA, where a difference in CPAP-users vs. non-users was evident. If age alone were driving the neuropathological changes it is unlikely that CPAP would have a beneficial effect. All of the neuropathological changes that correlated with age also had stronger relationships for CPAP non-users and weaker relationships for CPAP users, except GFAP-positive astrocytes in the subiculum where CPAP users had a stronger but non-significant relationship with ODI compared to the whole group. Further, many other measures in the present study were found to be independent of age, and consequently most of the increases in gliosis, hippocampal atrophy, demyelination and A β burden appear to be attributable to OSA severity.

6.6 Limitations

Due to the archival nature of the project there were several unavoidable limitations.

The sample size was relatively small. This limited the statistical power of the study, preventing the application of multivariate statistics and factor analysis. A larger sample would have enabled possible co-factors, such as BMI, to be controlled for and the individual contribution of age could have been determined. Also the influence of genes (eg ApoE) could not be adequately assessed with the present sample size due to the under-representation of some genotypes.

The sample may not be representative of the global population of OSA patients (or Australian patients), as all of the patients were from Iceland. Iceland differs from most other countries, including Australia, because of its cold climate and variable day length due to its northern location (64° latitude). Day length is particularly important in the present study because the circadian system responsible for the sleep-wake cycle is dependent on daylight. However, a study investigating the sleep patterns of people in Norway (69° latitude) and Ghana (5° latitude) has found no difference in total sleep time and sleep quality, measured across seasons (Friborg, Bjorvatn, Amponsah, & Pallesen, 2012). Iceland is less genetically diverse than other countries and the results of the present study may only be relevant to the Icelandic population. However, prevalence rates of OSA are similar throughout the world. A cohort study in Iceland estimated that the prevalence of moderate-severe OSA, defined by an AHI >15, is 19% of the Icelandic population (Arnardottir, Bjornsdottir, Olafsdottir, Benediktsdottir, & Gislason, 2016), this is comparable to a recent systematic review that found the prevalence of moderate-severe OSA (AHI >15) from 24 studies in 15 countries to be 6 – 17% in the general population, but as high as 49% in the elderly (Senaratna et al., 2017).

The lack of neuropsychological testing in the present study is a limitation. While none of the patients were diagnosed with AD or dementia, it is possible that some had undiagnosed cognitive deficits. Relationships between neuropsychological tests and structural brain changes have been investigated in OSA patients previously (Canessa et al., 2011).

Specifically, memory improvements after CPAP treatment are correlated with increased volume of the parahippocampal gyrus (Canessa et al., 2011). However, the hippocampus is involved in processing different types of memory and the present study has illustrated the regional variability of neuropathology within the hippocampus in OSA. Therefore, subtle changes on neuropsychological tests of specific types of memory might be correlated with neuropathological damage in specific regions of the hippocampus. If so, this might allow such neuropsychological tests to be used as ‘biomarkers’ that could predict the presence of neuropathology. Further, the impact of CPAP treatment on such relationships could be investigated. Future studies should include multiple neuropsychological tests to investigate this possibility.

The present study lacked an OSA-null group to control for the effects of age, lifestyle and comorbid factors. This may seem like a simple problem that could be overcome by obtaining autopsy tissue from age-matched individuals from Iceland. However, OSA is often undiagnosed and to establish a true control group, all participants must have undergone a PSG in order to confirm that they did not have OSA. Two participants in the current study were confirmed to have no OSA (ODI <5 events/h sleep), however this number is not sufficient for a control group. Future studies should be designed longitudinally in conjunction with a brain bank to ensure that PSG records are kept as part of the patients’ medical history.

Details of *post-mortem* interval were not known for the tissue used in this thesis. The effects of *post-mortem* interval on brain tissue have not been investigated thoroughly with most researchers utilising tissue with only a short *post-mortem* interval or matching post-mortem interval between experimental groups to control for these effects. However, it has recently been shown that phosphorylated tau, the main component of NFTs, remained stable in human hippocampus after a long post-mortem interval compared to a short *post-mortem* interval (Blair et al., 2016). Blair also found that GFAP remained stable after a long *post-mortem* interval, although previous studies have variously reported increased, decreased or unchanged GFAP staining with increasing *post-mortem* interval (ElHajj, Cachot, Müller, Riederer, & Riederer, 2016; Hilbig, Bidmon, Oppermann, & Remmerbach, 2004; K. H. C. Wu, Penfold, & Billson, 2002). Two of these studies were performed in mouse models and one utilised human retinal tissue. GS was found to be stable in *post-mortem* rat brain (T. Ritchie, Scully, de Vellis, & Noble, 1986). Other markers used in the present study have not been

investigated for *post-mortem* degeneration. The lack of consistent research on the effects of *post-mortem* interval, combined with the unknown *post-mortem* interval in the present study allows for the possibility that this may have influenced the results. Future studies controlling for the effects of *post-mortem* interval and investigating the effects of long-term *post-mortem* interval on human brain tissue are warranted.

The present study is limited in that only one measure of OSA severity was available. A more comprehensive investigation would include more than one measure of OSA severity and additional data from PSG recordings. This would enable a complete analysis of the specific sleep and oximetry variables to determine which neuropathological features are more related to sleep disturbance and hypoxia. Additionally, the use of ODI as a measure of OSA severity may be a limitation, as it is not the most common measure used. AHI is more widely used in the diagnosis of OSA (Gislason, 2014), however ODI and AHI are highly correlated (Ernst et al., 2016). Regardless, future studies should use multiple oximetry measures including time spent with an arterial saturation below 85% and nadir oxygen desaturation, as well as measures of sleep quality and quantity. Additionally, reports on subjective daytime sleepiness would be useful, particularly given that residual daytime sleepiness negates the benefits of CPAP treatment (Xiong et al., 2016).

The observational nature of the present study meant that adherence to CPAP could not always be verified. Patients who were classified as CPAP users had CPAP machines, however data regarding the nightly usage was not available in the present study. Further, due to missing data regarding CPAP use, there may have been patients included in the CPAP non-users group who had used CPAP at some stage in their lives.

The use of CPAP and the lack of knowledge about the consistency of CPAP use could have acted as a confounding variable for the analysis of the whole sample. Since CPAP use afforded an improvement in the neuropathological status of the patients, the fact that half the patient sample used CPAP would have decreased the likelihood of detecting the effect of OSA on neuropathology. Similarly, when the sample was separated into CPAP users vs. non-users, the sample size is halved and the statistical power available to find a true effect is reduced. These considerations imply that the neuropathological damage caused by OSA may be worse than what was detected in the present study, but that CPAP use confounded the

results. Future research with a larger sample and more accurate reporting measures for CPAP compliance could overcome this problem. With a large enough sample, CPAP use could be included as a co-factor using multiple regression analysis to determine the true extent of neuropathological damage in OSA while controlling for CPAP use.

The duration of undiagnosed and untreated OSA in the current sample is unknown. This is a problem for all investigations of OSA, but estimates can be obtained using questionnaires from patients and relatives about the age of onset of symptoms (eg. snoring, weight gain, daytime napping). Future studies should attempt to estimate the duration of OSA prior to diagnosis as this may be associated with the severity of neuropathology or the capacity of CPAP to reverse these features.

For each marker, analysis was restricted to a few sections of hippocampal tissue from archived blocks (due to the extremely limited amount of tissue available). This prevented stereological analysis of serial sections, or analysis of the complete hippocampus.

Furthermore the rest of the brain was not available, so it is not known whether the changes observed in the hippocampus are unique to this structure, or are present throughout the telencephalon. Despite these limitations, the present study has found several significant and important findings regarding the neuropathological changes that occur in the hippocampus of OSA patients.

6.7 Future research suggestions

Future research investigating neuropathology in OSA would ideally involve a much larger sample of patients who were diagnosed with OSA via PSG, including multiple oximetry and sleep variables. CPAP use would be measured objectively, via the data obtained from newer CPAP machines and they would be followed up at 5 year intervals with PSG recordings, neuropsychological testing and a sleepiness questionnaire. At autopsy the whole brain would be removed for use in a comprehensive neuropathological investigation that would use a wide variety of markers of cell injury to examine many brain structures. A control group of patients without OSA, confirmed by PSG, would also be included in the study. The increased number of participants and inclusion of a control group would provide the statistical power to

conduct multivariate analysis and control for additional factors such as age, BMI, *post-mortem* interval, co-morbidities, cognitive status and genotype. A longitudinal design such as this would enable a more complete understanding of the injuries caused to the brain by OSA.

Future studies should also investigate other aspects of neurodegeneration that were beyond the scope of the present project. Studies have shown that there are specific populations of wake-active neurons in the brainstem that degenerate with IH exposure (Y. Li et al., 2014; J. H. Zhang et al., 2010; Zhu et al., 2007; Zhu et al., 2016). It has been commonly reported that noradrenergic neurons of the LC degenerate in IH. Firstly, it is important to confirm these findings in the human brainstem in OSA. Secondly, these findings could be extended to investigate different neuronal populations in the hippocampus and basal forebrain. Specifically, the noradrenergic neurons that receive input from the LC neurons and the cholinergic neurons of the basal forebrain could be investigated, as both of these populations of neurons are known to degenerate in AD (Whitehouse et al., 1982; Zarow et al., 2003). Like in AD, individual populations of neurons may be differentially affected by OSA.

The exact mechanisms responsible for the neurodegenerative changes observed in OSA could not be determined in the present study due to the observational design. It is possible that oxidative stress mediates some of the injury in OSA, specifically NFT production and demyelination. Oxidative stress can lead to an increase in tau phosphorylation (Su et al., 2010) and the dysfunction of oligodendrocytes (Juurlink, 1997; Thorburne & Juurlink, 1996). Indeed, animal models have shown increases in oxidative stress markers in the brain after IH (Row, Liu, Xu, Kheirandish, & Gozal, 2003; Veasey et al., 2004; G. Zhan et al., 2005). While it has been suggested that oxidative stress is an important treatment target for OSA (J. Zhang & Veasey, 2012), markers of oxidative stress have not yet been investigated in the brains of OSA patients. Other interesting areas of investigation include the BBB and microvasculature of the brain. Both are reported to undergo degenerative changes in AD (Erickson & Banks, 2013; Hashimura, Kimura, & Miyakawa, 1991; Higuchi, Miyakawa, Shimoji, & Katsuragi, 1987; Wisniewski & Kozlowski, 1982), and hypoxia has been suggested to lead to BBB leakage and microvascular changes in OSA (D. C. Lim & Pack, 2014). These factors are also important to investigate in the context of CPAP treatment. It is important to determine which neuropathological features can be successfully treated with CPAP and which cannot, so that alternative treatments might be developed.

6.8 Conclusions

The present study has examined archived *post-mortem* tissue to evaluate a variety of neuropathological features in the hippocampus and brainstem of OSA patients. For the first time, neuropathological changes have been observed in the hippocampus of OSA patients. Further, most of these changes were found to be ameliorated with CPAP treatment.

OSA severity was associated with increasing burdens of NFTs and A β plaques in the hippocampus, but not in the brainstem; significant hippocampal atrophy, particularly in the CA1 but also in the dentate gyrus and entorhinal cortex; demyelination of the deep white matter in the entorhinal cortex; and regionally specific reactive gliosis throughout the hippocampus, except in the CA1. Similar neuropathological changes are characteristic of early AD, suggesting that both conditions share some common degenerative pathways. It is possible that the frequent hypoxic episodes and fragmented sleep in OSA trigger the unknown causative agent of AD and accelerate the pathological process.

CPAP was found to protect against many of the neuropathological changes observed. The present study provides support for the effectiveness of CPAP use against AD-like neuropathological changes in OSA. The burdens of NFTs and A β plaques, most neuroinflammation and some hippocampal atrophy were all decreased among patients that were known to have used CPAP. Conversely, the most severe neuropathology, similar to that seen in AD, was observed among CPAP non-users. The extent of demyelination and some hippocampal atrophy did not improve with CPAP use. Nonetheless, the majority of neuropathological damage related to increasing OSA severity was significantly reduced with CPAP use. This finding may have implications not only for the treatment of OSA patients, but also for the treatment of neurodegeneration and neuroinflammation in other conditions, including AD, by slowing or preventing the accumulation of AD-like pathology.

Patient age contributed to some of the present findings, particularly the atrophy of layer 2 of CA1, NFT burden and some reactive gliosis. Patient age was expected to be a confounding variable, given that neurodegeneration is common in the ageing brain; however most of the

neurodegeneration observed in the present study occurred independently of age, implying that degeneration associated with OSA exceeds that due to age alone.

The present study observed several of the neuropathological hallmarks of AD in the brains of OSA patients, and these were found to increase with increasing OSA severity. As well as making a valuable contribution to the characterisation of the neuropathology of OSA, the present findings provide compelling evidence for the hypothesis that the presence of OSA accelerates the pathogenesis of AD. Additionally, the protective effect of CPAP has implications for current OSA patients who may be reluctant to use their CPAP devices, and it raises the interesting possibility that CPAP could be used in the future as a prophylactic treatment for AD.

Chapter 7 References

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Chapter 8 Appendices

Appendix A: National Bioethics Committee of Iceland ethics approval

RMIT University
Dr. Steven Robinson, Professor



Reykjavík 28. febrúar 2017
Tilv.: VSNb2009080006/03.01

Efni: Regarding ref. number: 09-087 - CM
English: Brain and liver tissue changes in obstructive sleep apnea
Icelandic: Taugavefsskemdir í kæfisvefni

Enclosed is a copy of the original approval by the National Bioethics Committee, Iceland, dated 23 February 2010 (CM in English dated 16 February 2016), regarding the above mentioned research application submitted to the Principal Investigator, Þorarinn Gíslason, dr. med., University Hospital Iceland.

Certain irregularities have been brought to our attention concerning the execution of the approved protocol, especially regarding the use of tissue samples abroad. We've investigated those irregularities and introduced amendments to the procedures of the Icelandic Biobank in order to secure that this will not happen again.

We've come to the conclusion that none of those incidents should have to interfere with your work or the work being conducted by your group or of your PhD students.

We therefore approve that you make use of this original Confirmation of approval to finish your work and make it possible for your PhD students to finish their thesis and the defence thereof. We do however request that no further work will be carried out on those samples, without new application/approval and that the rest of the samples will be returned to Iceland as soon as possible and no later than one year after the disputation of the latter PhD student concerned.

Sincerely Yours,
Kristján Erlendsson, MD

Chairman of the National Bioethics Committee of Iceland

Appendix B: RMIT ethics approval



College Human Ethics Advisory Network (CHEAN)
College of Science, Engineering and Health

Email: seh-human-ethics@rmit.edu.au
Phone:

5 May 2017

Professor Stephen Robinson
School of Health and Biomedical Sciences
RMIT University

Dear Prof Robinson

ASEHAPP 71-16 Neurodegenerative changes in obstructive sleep apnea

Thank you for providing current institutional approval as requested and I am pleased to inform you that you have met the condition for the approval of your application. Your application is now approved for a period of **3 Years** from the date of this letter to **31 December 2020** and your research may now proceed.

The CHEAN would like to remind you that:

All data should be stored on University Network systems. These systems provide high levels of manageable security and data integrity, can provide secure remote access, are backed up on a regular basis and can provide Disaster Recover processes should a large scale incident occur. The use of portable devices such as CDs and memory sticks is valid for archiving; data transport where necessary and for some works in progress.

The authoritative copy of all current data should reside on appropriate network systems; and the Principal Investigator is responsible for the retention and storage of the original data pertaining to the project for a minimum period of five years.

Please Note: Annual reports are due on the anniversary of the commencement date for all research projects that have been approved by the CHEAN. Ongoing approval is conditional upon the submission of annual reports failure to provide an annual report may result in Ethics approval being withdrawn.

Final reports are due within six months of the project expiring or as soon as possible after your research project has concluded.

The annual/final reports forms can be found at:

www.rmit.edu.au/staff/research/human-research-ethics

Yours faithfully,

**Associate Professor Barbara Polus Chair,
Science Engineering & Health College
Human Ethics Advisory Network**

Cc Student Investigator/s: Ms Jessica Owen, School of Health & Biomedical Sciences

Ms Cuicui Xu, School of Health & Biomedical Sciences

Other Investigator/s: Prof Jiming Ye, School of Health & Biomedical Sciences